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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
TBA 10/019386

INTERNATIONAL APPLICATION	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/10509	19 April 2000	30 June 1999

TITLE OF INVENTION
DNA BINDING PROTEIN AND SEQUENCE AS INSULATORS HAVING SPECIFIC ENHANCER BLOCKING ACTIVITY FOR REGULATION OF GENE EXPRESSION

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a. ☐ is transmitted herewith.
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☐ A translation of the International application into English (35 U.S.C. 371(c)(2)). with oath

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (executed)

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included.

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or Information:

- Copy of Written Opinion
- International Search Report
- Preliminary Examination Report
- Information Concerning Elected Offices Notified of Their Election
- Copy of International Application Published Under the Patent Cooperation Treaty (PCT)
- Notification Informing Applicant of the Communication of the International Application to the Designated Offices
- Copy of PCT Request(6 pages)
- Notification of Receipt of Record Copy
- Notification Concerning Submission or Transmittal of Priority Document
- Return postcard.

531 Rec'd PCT/F 28 DEC 2001

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NO.
TBA 10/019386	PCT/US00/10509	2026-4297US

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ... $890.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO $740.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33 (1) - (4) $710.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) - (4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00
Surcharge of **$130** for furnishing the oath or declaration later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	33-20	13	X $18.00	$234.00
Independent claims	6-3	3	X $84.00	$252.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$280.00

TOTAL OF ABOVE CALCULATIONS =	$1656.00
Reduction of ½ for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).	$ 0.00
SUBTOTAL =	$.00
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$ 0.00
TOTAL NATIONAL FEE =	$.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property =	$ 0.00
TOTAL FEES ENCLOSED	$1656.00
Amount to be refunded:	$
charged	$

a. ☐ A check in the amount of $ cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 13-4500 in the amount of $ 1656.00 to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-4500, ORDER NO. 2026-4297US . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Title Of The Invention

DNA BINDING PROTEIN AND SEQUENCE AS INSULATORS HAVING SPECIFIC ENHANCER BLOCKING ACTIVITY FOR REGULATION OF GENE EXPRESSION

Field Of The Invention

The present invention relates to the identification of an insulator nucleic acid sequence which has the ability to block the action of enhancers and prevent gene activation, and a DNA binding protein which binds to the insulator sequence. The invention also relates to methods for insulating the expression of a given gene by employing the insulator sequence and/or the DNA binding protein of the invention. The invention further relates to the identification of an insulator element which has the ability to block the expression of the insulin growth factor 2 (Igf2) gene. The enhancer-blocking activity of this insulator element is dependent upon CTCF binding to the insulator. Methylation of the insulator element abolishes the ability of the CTCF to bind to the insulator and would therefore result in loss of CTCF-dependent enhancer-blocking activity. The invention also relates to methods of modulating the enhancer-blocking activity of the insulator element.

Background Of The Invention

Enhancer-mediated activation is a fundamental mechanism of gene regulation in eukaryotic organisms. Enhancers can act over large distances to activate transcription, independent of their orientation and position relative to the promoter. In many cases, if given access, enhancers can act promiscuously to activate transcription of heterologous promoters. In fact, some types of cancers are thought to arise as a result of translocations which artificially juxtapose an oncogene with a heterologous enhancer.

Genome sequencing has revealed many cases where differentially regulated genes neighbor each other at distances over which enhancers could act, yet the genes are independently regulated. Thus, mechanisms are likely to exist that are able to prevent the action of an enhancer on a neighboring locus. This restriction must be achieved, at least in some cases, without impeding the action of the enhancer within its native locus. A DNA element able to function in this way

would, in effect, constitute a boundary to the action of an enhancer, thereby preventing it from acting across the boundary, while otherwise leaving the enhancer unimpeded. This property is one of the defining characteristics of an insulator, a type of regulatory element that has only recently been recognized. (Kellum and Elgin, 1998; Udvardy, 1999; Bell and Felsenfeld, 1999, U.S. Patent No. 5,610,053 to J. Chung et al.).

The first DNA sequences to be described as having the properties of an insulator were the *scs* and *scs'* elements of *Drosophila*, which were initially identified as marking the chromatin boundaries of a heat shock locus. When *scs* elements (i.e., *scs* DNA sequences) were placed on either side of a gene for eye color and introduced as transgenes into *Drosophila* embryos, the resulting offspring flies all had similar eye color, independent of the site of integration of the transgene. This result indicated that *scs* had protected the reporter gene from both negative and positive endogenous influences, or 'position effects' (Kellum and Schedl, 1991 and 1992). Another *Drosophila* insulator element, *gypsy*, was first identified because of its ability to block the action of an enhancer on a promoter when the element lay between them, but not otherwise (Holdridge and Dorsett 1991; Geyer and Corces, 1992; Dorsett, 1993). Studies of these elements have led to a working definition of an insulator as an element that is capable of protecting against position effects and/or blocking enhancer action in a directional manner. For both scs' and gypsy, proteins have been identified that bind specifically to the DNA elements and are, at least in part, responsible for mediating insulator activity (Geyer and Corces, 1992; Zhao et al., 199S).

Insulator elements have also been identified in vertebrates (Chung et al., 1993 and 1997; Zhong and Krangel, 1997; Robinett et al., 1997). U.S. Patent No. 5,610,053 to Chung et al. has described a 1.2 kb DNA insulator element, which was derived from the 5' end of the chicken ß-globin locus and exhibited strong enhancer-blocking activity. (Chung et al., 1993 and 1997). This region contains a constitutive DNase I hypersensitive site that is present in all tissues. The 1.2 kb insulator element coincides almost exactly with the point of transition between an active chromatin conformation, marked both by histone hyperacetylation and a

heightened general sensitivity to DNase, and an inactive domain extending farther 5' that is insensitive to nuclease and less highly acetylated. (Hebbes et al., 1994).

Within the 1.2 kb element is a 250 base pair (bp) 'core' fragment or region that possesses a large part of the enhancer blocking activity (U.S. Patent No. 5,610,053 to J. Chung et al.; Chung et al., 1997). However, the identification and characterization of additional and specific sub-sequences having insulator function within the 1.2 kb insulator element and 250 bp core remain to be elucidated. In addition, there remains to be discovered and identified one or more DNA binding sites within the core region that is/are necessary and sufficient for enhancer-blocking activity and that are recognition sites for regulatory protein binding to DNA.

Directional enhancer-blocking activity of proteins that bind to specific insulator nucleic acid sequences provides to the art important methods to control gene function at numerous complex gene loci in many organisms. The identification and characterization of an insulator sequence and protein that binds thereto can establish the foundation for maintenance of boundaries between different groups of genes that have distinct regulatory patterns. The use of such isolated sequences and their purified binding proteins provide significant tools for the regulation of gene expression and function in mammals and plants.

Description Of The Drawings

Figs. 1A-1G show the fine mapping of the insulator core. In Figs. 1A and 1B, the position of HS4 was measured by comparing the migration of the DNase I digestion fragment generated by limited digestion of chicken erythrocyte chromatin to the migration of DNAs of known length and identical composition. The logic of this mapping is outlined in Fig. 1A. Fig. 1B shows the autoradiographic results of enhancer blocking activity of fragments of the core. The position of the hypersensitive site relative to previously defined DNase I footprints (Chung et al., 1997) is indicated in Fig. 1C. Figs. 1D-1F show the results of enhancer blocking assays in which the elements indicated were placed between enhancer and promoter as depicted in Fig. 1G, and the relative number of neomycin-resistant colonies was counted. A schematic of each inserted element is shown

("Test Fragments"), as well as the relative numbers of neomycin-resistant colonies observed ("Relative Neo^R Colonies"), and the numerical value of the insulation effect ("Fold Insulation") relative to the non-insulated controls (pNI and λ DNA). In particular, Figs. 1D and E show the effect on enhancer blocking of deletion of footprinted regions from the core. Fig. 1F shows increased enhancer blocking that is observed when insulating elements were multimerized. The data presented in Figs. 1D-F represent an average of at least 4 independent assays. Fig. 1G presents a schematic diagram of the construction used to test various DNA fragments for enhancer-blocking activity.

Figs. 2A-2E present the identification of a minimal enhancer blocking site. In Fig. 2A, a 90 bp fragment spanning FII and FIII was subjected to further deletion; the effects on enhancer blocking are shown. Fig. 2B presents the results of an examination of the effect of the relative positions of enhancer and promoter on the enhancer-blocking effect of FII in the colony assay. Fig. 2C presents the effect of mutation of Sp1 sites within FII/III on enhancer blocking. Fig. 2D shows the results of an evaluation of the contribution to the enhancer blocking activity of FII of motifs homologous to α2 and Su(Hw). Fig. 2E shows a truncated sequence of FII (SEQ ID NO:2) and its homologies to known transcription factor binding sites, namely, Su(Hw): SEQ ID NO:3; Sp1; SEQ ID NO:4; and α2: SEQ ID NO:5. The enhancer-blocking data presented in Figs. 2A-D represent the average of 2-5 independent assays for each construction.

Figs. 3A-3D show the sequence specificity of enhancer blocking and nuclear factor binding by FII. In Fig. 3A, the indicated sequences (SEQ ID NOS:1, 6-10) were inserted into the AscI site of pNI; their ability to block an enhancer was measured in the colony assay. Specifically, as shown in Fig. 3A, SEQ ID NO:1 corresponds to the FII fragment; SEQ ID NO:6 corresponds to x5'; SEQ ID NO:7 corresponds to xM; SEQ ID NO:8 corresponds to x3'; SEQ ID NO:9 corresponds to ΔF; and SEQ ID NO:10 corresponds to rev sequences. Figs. 3B and 3C show gel mobility shift assays with a labeled 60 bp FII probe and nuclear extracts from human K562 (Fig. 3B) and chicken red blood cells (RBC), (Fig. 3C). Cold competitors as shown (sequences in Fig. 3A) were added at a 100 fold molar excess

in these experiments. Fig. 3D presents a comparison of the capacity of the indicated FII mutants (SEQ ID NOS:1, 6-17) to (i) act as insulators in the colony assay, (ii) bind to a candidate insulator protein in gel shift and (iii) bind to CTCF in southwestern binding assays (see also Figs. 4A-C). Data were normalized with FII activity considered as 100% in each assay.

Figs. 4A-4D relate to purification of an FII binding factor. Fig. 4A shows sequence specific FII binding observed at ~140 kDa apparent molecular weight in protein fractions obtained during different stages of purification. Fig. 4B shows a schematic outline of the protocol used to purify the FII binding factor. Figs. 4C and 4D provide a representative example of a Coomassie stained gel of the purified fractions eluted from the hydroxyapatite column with the internal peptide sequences obtained from the indicated band indicated (labeled "Coomassie", Fig. 4C), and the result of a southwestern assay of FII binding to this fraction (labeled "Southwestern", Fig. 4D).

Fig. 5 shows FII binding and enhancer blocking by CTCF. Purified FII binding factor (lane 1) and *in vitro* translated CTCF (lanes 2-11) have identical specificity for FII (compare to Fig. 3A) and identical complex migration in a gel shift assay when bound to either FII (lanes 2-8) or previously-characterized CTCF sites from the chicken *c-myc* promoter (lane 9), the chicken *lysozyme* promoter (lane 10) or the human *amyloid beta-protein* promoter (lane 11). The table in the right of the figure summarizes the capacity of CTCF sites to act as enhancer-blockers in the colony assay. The data presented represent the average of two independent measurements.

Figs. 6A-6C show sequence homologies among CTCF sites and vertebrate insulators. Fig. 6A, (SEQ ID NOS:1, 8, 18-20), shows that the alignment of FII with other known CTCF sites reveals a conserved 3' region which corresponds to the sequence altered in the x3' mutant (see Figs. 3A-3D). Fig. 6B, (SEQ ID NOS:1, 21-27), shows the alignment of the 100 bp repeats of the *Xenopus* RO element and FII. Fig. 6C, (SEQ ID NOS:1 and 28), shows the alignment of FII with a homologous site (BEAD-A) in the BEAD-1 element from the human T cell receptor α/δ locus.

Figs. 7A and 7B show the conservation of sequence-specific enhancer blocking activity among vertebrate insulators. Fig. 7A presents gel mobility shift assays with FII and BEAD A as probes to reveal sequence specific binding to partially purified CTCF. An antibody raised against a C-terminal peptide of CTCF specifically supershifts both complexes. Fig. 7B shows enhancer blocking activities of vertebrate insulators. These data are the average of at least two independent experiments, with the exception that the data for the RO element are from a single determination.

Figure 8A-B show that differentially methylated region (ICR) upstream of *H19* has the enhancer-blocking properties of an insulator. Fig. 8A shows a schematic of the neighboring mouse *Igf2* and *H19* genes. On the maternally inherited chromosome, the ICR is unmethylated (white rectangles) and contains two nuclease-hypersensitive regions (hatched boxes, HS1 and HS2); on the paternally inherited chromosome, the ICR is methylated (black rectangles) and contains no hypersensitive sites. Deletion of a 1.6 kb fragment of the ICR (termed the DMD fragment) eliminates HS2 and most of HS1. Fig. 8B shows enhancer-blocking activity of various constructs. Constructs in which various fragments of the ICR were inserted at defined positions relative to the enhancer and promoter were prepared. For each construct, colony number was normalized to an uninsulated control, NI. Data are the average of three independent measurements.

Figure 9A-D shows conserved CTCF sites within the *H19* ICR. Fig. 9A shows sequences of the CTCF sites clustered upstream of the mouse, rat, and human *H19* genes. Shading indicates identity among the sites; gray shading indicates species-specific identities, while black shading indicates cross-species sequence conservation among these sites. Fig. 9B shows enhancer-blocking activity of a fragment spanning only m3 from the mouse ICR. Data are the average of three independent experiments. Fig. 9C shows gel mobility-shift analysis of β-globin FII (60-mer) and 83-91-mer duplexes spanning mouse and human ICR sites binding to K562 nuclear extract (E), partially purified (chicken) CTCF (P), and *in vitro* translated human CTCF (I). An asterisk indicates the position of the CTCF:DNA complex. Labeled DNA probes are indicated at the panel bottom. Fig. 9D shows analysis of CTCF binding to representative mouse and human ICR sites. DNAs

were incubated with K562 nuclear extract in the presence of a 50-fold excess of unlabeled competitors as indicated or with an anti-CTCF antibody. SS indicates the position of the supershifted CTCF complex.

Figures 10A-B show that CTCF is responsible for the methylation-sensitive enhancer-blocking activities of the mouse and human ICRs. Fig. 10A shows enhancer blocking activities of fragments of the mouse ICR after sequential deletion of the sequences spanning individual CTCF sites. Results are the average of two to three independent measurements. Fig. 10B shows on the left panel the effect of CpG methylation on binding of partially purified chicken CTCF to various sites in the absence of competitor DNA (-), in the presence of 50-fold excess of unlabeled duplex DNA (S, self) or a 50-fold excess of unlabeled duplex of identical sequence with ^{5me}C incorporated at every CpG (M, uniformly methylated). Labeled DNA probes are indicated at the bottom of the panel. In the right panel: the effect of ^{5me}C substitution at a single site (M1, singly methylated at the first CpG in the black-shaded region of Fig. 9a).

Figure 11 shows a model for methylation-dependent modulation of insulator action in the epigenetic regulation of *Igf2*. On the maternally inherited chromosome, the ICR is unmethylated. This allows binding of CTCF to its sites (m1-4), two in each nuclease-hypersensitive region (shaded boxes), and the resulting insulator blocks activation of the maternal copy of *Igf2* by the *H19* enhancer. On the paternally inherited chromosome, the ICR is methylated. This prevents CTCF binding, thereby inactivating the insulator and allowing the *H19* enhancer to activate *Igf2*.

Summary Of The Invention

The present invention provides a newly-identified insulator nucleic acid sequence that acts as a barrier to the influences of neighboring *cis*-acting elements, thereby preventing gene activation, for example, when juxtaposed between an enhancer sequence and a promoter sequence. According to the present invention, the new insulator nucleic acid sequence is 42 base pairs (bp) in length and comprises a new, specific and previously unidentified fragment of the chicken beta (β)-globin insulator element. This insulator sequence was shown to be both

necessary and sufficient for the enhancer blocking activity in human cells, as described herein.

It is an object of the present invention to provide a method for using the newly-characterized and isolated insulator element to insulate or buffer the expression of a reporter gene from adverse effects of neighboring or surrounding chromatin. The incorporation of the defined insulator sequence into vectors and constructs allows gene transfer and expression in cells and tissues with virtually no concern for suppression or inhibition of expression due to the chromosomal milieu after integration.

It is another object of the present invention to provide genetic expression constructs or vectors which are designed to contain one or more operational DNA sequence insulator elements comprising SEQ ID NO:1 which can insulate or buffer the activity of a particular gene from the effects of the activity of *cis*-acting regulatory elements, such as enhancer or silencer regions of the DNA. The constructs may contain one or more insulator elements and one or more reporter genes in the form of transcription units or mini-loci, including at a minimum, an enhancer, a promoter, and a reporter gene. The insulator element-containing constructs allow for the transfection of cells of a particular lineage or of a particular tissue type, depending upon the gene to be transfected and upon other features of the construct which may be cell- or tissue-specific, such as specific promoter or enhancer elements, or upon particular regulatory molecules, proteins, or factors which are produced by a particular cell or tissue type and which influence the expression of a given transfected gene.

In accordance with the invention, the insulator element(s), reporter gene(s), and transcription unit may be provided in the form of a cassette designed to be conveniently ligated into a suitable plasmid or vector, which plasmid or vector is then used to transfect cells or tissues, and the like, for both *in vitro* and *in vivo* use.

It is a further object of the present invention to provide a mechanism and a tool to restrict the action of *cis*-acting regulatory elements on genes whose activities or encoded products are needed or desired to be expressed in certain cells and tissues. The genes to be insulated and expressed may be introduced into cells by employing the constructs or vectors achieved by the present invention in which

one or more insulator elements in a chromatin domain are strategically positioned so as to buffer the transfected genes from the influence of the action of other DNA sequences from different chromatin domains located in *cis*.

It is also an object of the present invention to provide a specific binding site for a purified protein, CTCF, which is an eleven zinc finger DNA binding protein, highly conserved in vertebrates. The sequence specificity of CTCF accounts precisely for the sequence requirements of directional enhancer-blocking *in vivo*.

It is a further object of the present invention to provide a method for insulating a given gene by employing the insulator sequence and/or the CTCF binding protein to achieve directional enhancer blocking of a gene.

Also provided is a kit or kits containing the vector constructs of the invention and used to insulate the expression of a heterologous gene or genes integrated into host DNA.

The invention further provides a method and constructs to insulate the expression of a gene or genes in transgenic animals such that the transfected genes will be able to be protected and stably expressed in the tissues of the transgenic animal or its offspring, for example, even if the DNA of the construct integrates into areas of silent or active chromatin in the genomic DNA of the host animal.

Yet another object of the present invention to provide a method for insulating the expression and function of a given gene by employing the DNA binding protein CTCF to bind to the insulator sequence as described herein.

The invention further relates to the identification of an insulator element which has the ability to block the expression of the insulin growth factor 2 (Igf2) gene. This insulator element contains CTCF binding sites and its enhancer-blocking activity is dependent upon CTCF binding to these sites. Methylation of the insulator element abolishes the ability of the CTCF to bind to the insulator and would therefore result in loss of the CTCF-dependent enhancer-blocking activity.

The invention also relates to methods of modulating the enhancer-blocking activity of an insulator by targeted methylation or demethylation of the insulator.

Further objects and advantages of the present invention will be appreciated in light of the description herein.

Detailed Description Of The Invention

The present invention provides an isolated, 42 base pair (bp) fragment (DNA sequence motif) of the chicken ß-globin insulator which has been newly found to be both necessary and sufficient for enhancer blocking activity in human cells. This DNA fragment, called FII herein, has been found to serve as an insulator molecule, i.e., a DNA sequence which can act as a barrier to the influences of neighboring *cis*-acting elements, for example, to prevent gene activation when located between an enhancer and a promoter of a given gene.

According to the present invention, this small DNA sequence motif, FII, comprises the minimal binding site for a cellular DNA binding protein and has the following DNA sequence, from 5' to 3' :

5' - C C C A G G G A T G T A A T T A C G T C C C T C C C C C G C T A G G G G G C A G C A - 3' (SEQ ID NO:1), (Figs. 3A, 3D and 6A-6C).

Newly identified and isolated in accordance with the present invention, the sequence motif of SEQ ID NO:1 accounts for most of the ability of the insulator element to block enhancer activity. Indeed, the function of this smaller sequence has been shown to be pivotal to the ability of the insulator element to block the action of enhancers.

The 42 bp fragment containing the sequence motif of SEQ ID NO:1 was able to suppress enhancer activity in a directional manner about as well as the full 1.2 kb element as described in U.S. Patent No. 5,610,053 to J. Chung et al., the contents of which are hereby incorporated by reference herein. The fragment also contains binding sites for Sp1 and the yeast α2 repressor; however, mutation of these sites had no effect on its blocking activity. By contrast, mutations of the 3' end of FII site did abolish enhancer-blocking.

According to the present invention, a DNA fragment encompassing SEQ ID NO:1 has also been newly discovered to be the core binding site for CTCF, a DNA binding protein that is highly conserved in vertebrates. A further significance of the CTCF site is that it has been found within the BEAD 1 element

of the T-cell α/δ locus (Zhong and Krangel, 1997) and accounts for the activity of the BEAD 1 element. The DNA sequence motif characterized by SEQ ID NO:1 specifies the minimal functional binding site for CTCF, as demonstrated in *in vitro* studies. For optimal binding by the CTCF protein, the DNA sequence motif characterized by SEQ ID NO:1 preferably comprises additional base pairs at the the 5' and 3' ends, thereby yielding a DNA binding element having approximately 50 to 70, more preferably 50-60 total base pairs, which includes SEQ ID NO:1. For example, the DNA binding element for CTCF may comprise SEQ ID NO:1 and from about 5-20 additional bases, added to the 5' and 3' ends of the sequence, without adversely affecting its insulator function.

When the 42 bp FII DNA fragment was used as a probe in gel retardation experiments, two major shifted bands were observed. One of these bands was attributable to interaction with Sp1. The other band appeared to be associated with insulating activity, based on its properties determined from competition experiments. More specifically, the second of the two bands was competed by any DNA that was also active in the enhancer blocking assay, but not by any of the inactive mutated sequences that were tried. Similar gel shift patterns were obtained with extracts from nuclei from chicken erythrocytes and from the human erythroleukemia cell line, K562, which were used to carry out the enhancer blocking assays. These observations and results were used to purify the protein responsible for the specific shifted band. The purified protein was determined to be the protein CTCF. The product of an *in vitro* transcription/translation reaction with cloned CTCF cDNA yielded results that were identical to those obtained using the above-mentioned cell extracts.

CTCF is an 82 kDa protein with 11 zinc fingers (Filippova et al. 1996), and is characterized by an unusually extensive DNase I footprint (51 bp) when bound to its site on DNA, consistent with an involvement of several fingers in typical binding sites. It migrates aberrantly on acrylamide/SDS gels, which accounts for the discrepancy in apparent molecular weight (Klenova et al., 1997). Studies of CTCF in other systems suggest that it can play a variety of regulatory roles. For example, it binds to the promoter of the amyloid β-protein precursor and causes transcriptional activation (Vostrov and Quitschke, 1997), but when it

interacts with sites in the *c-myc* oncogene, it causes repression (Filippova 1996). CTCF has also been found to be capable of acting in synergy with certain thyroid hormone receptor binding sites both in repression and in T3 induction (Baniahmad et al., 1990). Not all of the 11 zinc fingers of the protein are involved in binding to the sites that have been examined so far. Furthermore, different sites employ partially different subsets of fingers to contact the DNA (Filippova et al. 1996). From this, it can be surmised that the characteristics of the binding site would have a large influence on the conformation of the protein, the nature of its interactions with cofactors, and its ultimate biological effect(s).

According to the results presented herein, the CTCF binding site is necessary and sufficient for enhancer blocking activity, as demonstrated in the exemplified assays. The presence of similar binding sites at each of the vertebrate loci known to have enhancer-blocking activity is strong evidence for the role of CTCF sites in insulator function *in vivo*.

Also according to the present invention, the CTCF binding site has been determined to be a sub-fragment (42 bp) of the larger 1.2 kb insulator element containing the β-globin 5' HS4. As mentioned above, it is noteworthy that the β-protein 5' insulator element shares with the *Drosophila* insulators the additional ability to protect against position effects. For example, when two copies of the entire 1.2 kb fragment containing the β-globin 5' HS4 are placed on either side of a stably-integrated reporter gene, the reporter is protected both against variation in expression from one line to another and also against extinction of expression over a period of at least 40-80 days in culture (Pikaart et al., 1998). It is likely that this activity depends upon sequences other than or in addition to FII within the larger insulator element. The complete activity of the β-globin 5' insulator element is thus likely to involve multiple components.

It has been newly determined as described herein that enhancer blocking activity of the 5' β-globin insulator and the isolated portions thereof, is dependent upon CTCF. In addition, similar DNA binding site sequences are present in two other vertebrate insulators. The first of these is the BEAD-1 element found in the human T-cell receptor (TCR) α/δ locus (Zhong and Krangel, 1997). BEAD-

1, which has strong directional enhancer blocking properties, is located between TCR δ gene segments and TCR α joining gene segments. It has been proposed that BEAD-1 prevents a δ-specific enhancer from acting on the α genes early in T-cell development. The present findings have shown that BEAD-1 contains a CTCF binding site (i.e., the 42 bp DNA sequence motif sub-fragment of the 1.2 kb element) and that this site is responsible for a large portion of the observed enhancer blocking activity.

Taken together, the results described herein suggest a conserved and perhaps widely used function of insulators in which CTCF is involved in the maintenance of distinct regulatory regions. Indeed, additional analyses in the inventors' laboratory have shown that the 3' end of the chicken β-globin locus is marked by a hypersensitive site with similar properties to 5' HS4. This 3' end hypersensitive site also contains a CTCF binding site. This element is located between the globin genes and a nearby, yet distinctly unrelated, gene encoding an odorant receptor (Burger et al., 1999), further substantiating the nature and likely function of these boundary elements *in vivo*.

Insulators typically are capable of both blocking enhancer activity and protecting against position effects. These two functions might have only partially overlapping mechanisms. Protection against position effects implies that activation by external endogenous enhancers is blocked, consistent with the activity described herein. However, position effects also arise from silencing induced by neighboring heterochromatin. While the insulator described herein is able to protect against external position effects, it may also be that additional components of the insulator element, or additional cofactors, are involved in protecting against such effects.

Without wishing to be bound by theory, it may be likely that in some situations, where enhancer blocking activity is all that is required, a CTCF binding site alone is sufficient, while in the case of a permanent chromatin domain boundary, such as that found at the 5' end of the chicken β-globin locus, additional components are involved. Indeed, even in those cases where only CTCF sites are present, the activity of CTCF may require the participation of other proteins, just as

the directional enhancer-blocking activity of the suppressor of Hairy-wing protein involves interaction with the mod(mdg4) protein (Gerasimova et al., 1995; Gdula et al., 1996; Georgiev and Kozycina, 1996; Gdula and Corces, 1997; Gerasimova and Corces, 1998). Thus, proteins that interact with, or bind to, CTCF are likely to exist, although none are presently known.

According to the present invention, an insulator element, or CTCF binding site, is preferably located between an enhancer and a promoter to influence expression. The position of the insulator is the determining factor -- it can be inserted in either orientation with equal effect and insulator function. With regard to current understanding of how enhancers function, various models have been proposed to account for enhancer blocking. The models fall into two broad categories: steric models and tracking models (Kellum and Elgin, 1998; Udvardy, 1999; Bell and Felsenfeld, 1999). Steric mechanisms postulate that insulators partition an enhancer and a promoter into two separate domains that are inaccessible to each other. The steric models are related to existing ideas of how enhancers work. There is strong evidence that enhancers recruit the RNA polymerase complex to the promoter through interactions between proteins bound to that complex and proteins bound to the enhancer. If this occurs through formation of a loop between the enhancer site and the promoter, then the enhancer will be blocked if looping is prevented. Tracking models presume that some activating signal must travel along the DNA from enhancer to promoter, and that the insulator blocks this transmission. Such activating signals might involve replication, or might for example, require that a polymerase complex travel along the DNA to reach the promoter. The identification of CTCF as a vertebrate enhancer blocking protein provides the ability to functionally dissect the enhancer blocking process.

CTCF is likely to play a role in the function of many insulator elements. The first vertebrate insulator to be identified was located at the 5' end of the chicken β-globin domain; this 5' insulator site is likely to serve to protect the globin genes from inappropriate interaction with neighboring genes and their regulatory elements (U.S. Patent No. 5,610,053 to J. Chung et al.). In particular, an independently regulated gene coding for a folate receptor has recently been identified 5' of the globin locus. The globin and folate receptor genes are close

enough so that the regulatory elements of the two loci might influence each other inappropriately in the absence of an insulator. A similar situation exists in the case of the T-cell receptor locus (Zhong and Krangel, 1997), where the BEAD insulator element shields against inappropriate activity of an enhancer. The presence of CTCF DNA binding sites in these quite different genetic loci implies that the role of such sites in the establishment and maintenance of enhancer boundaries is likely to be a conserved and important component of gene regulation.

According to the present invention, the insulator element of SEQ ID NO:1 demonstrates enhancer-blocking function, both by itself and when bound by the CTCF protein as described herein. Thus, this element and the CTCF protein can be regarded, in a broad sense, as a receptor and its ligand. These two entities can be used together or separately to regulate gene expression. The insulator defined herein is a DNA sequence which is capable of acting as a barrier to neighboring *cis*-acting elements, insulating the transcription of a gene placed within its range of action, when juxtaposed between an enhancer and a promoter. Gene activation by external endogenous enhancers is blocked when the insulator is positioned between the enhancer and the promoter of a given gene.

A significant advantage of the insulator sequence defined by SEQ ID NO:1 is that it is a small molecule and is more versatile for use in a variety of vectors for gene delivery into cells and organisms. By contrast, the larger 1.2 kb insulator and 250 bp core sequences are cumbersome and their sizes may preclude their use in some applications of gene delivery and/or gene transfer. Indeed, according to the results herein, the DNA motif which comprises the insulator of the present invention has been found to be both necessary and sufficient for insulating and enhancer-blocking effects and so may be preferentially used as the insulator of choice in the vectors and constructs embraced by the invention.

Another aspect of the insulator sequence described herein, or the insulator bound by its cognate DNA binding protein, is the protection of a stably integrated reporter gene from position effects.

In one embodiment, the present invention provides constructs or vectors containing the insulator sequence of SEQ ID NO:1, enhancer and promoter sequences, and at least one heterologous gene sequence encoding a protein,

polypeptide, or peptide, wherein the insulator sequence is situated between the enhancer and promoter sequences, and upstream of the gene sequence. The construct or vector provides the vehicle for introducing the heterologous gene into a cell where it is integrated into the DNA and expressed and where its expression is insulated from the unwanted or adverse effects of *cis*-acting elements or sequences in surrounding chromatin. Insulated gene expression and regulation of gene expression by the use of the insulator of the present invention can be further regulated or controlled by endogenous CTCF, or a CTCF-like protein which binds to the DNA binding site specified by SEQ ID NO:1. Alternatively, a gene encoding CTCF, or a gene encoding a protein having DNA binding function like CTCF, can be used in a vector or construct that is co-introduced into a cell and expressed to more precisely control the expression of the introduced heterologous gene.

The vectors or constructs as used herein broadly encompass any recombinant DNA material that is capable of transferring DNA from one cell to another. The vector as described in the above embodiment can represent a mini-locus which can be integrated into a mammalian cell where it can replicate and function in a host cell type-restricted and copy number dependent manner, independent of the site of integration. Thus, the expression and production of the introduced gene is insulated from any effects exerted by neighboring genetic loci or chromatin following integration.

The insulator element as described herein can be employed to provide novel constructs for the efficient isolation and protection of genes and for the undisturbed production of a particular protein or other molecule encoded by a gene used in the constructs introduced into cells. The insulator element of the invention may also be used to insulate particular genes introduced and subsequently expressed in transgenic animals, such as fruit flies (e.g., *Drosophila melanogaster*), mice, rats, rodents, higher mammals and the like. Constructs containing the insulator element of the invention may be introduced into early fetal or embryonic cells for the production of transgenic animals containing the functional insulator element and reporter gene transcription unit. By insulating a gene or genes introduced into the transgenic animal, the expression of the gene(s) will be protected from negative or inappropriate regulatory influences in the chromatin at or near the

site of integration. In addition, the insulator will prevent inappropriate or unwanted activity from external enhancers that may affect the expression of the gene that has integrated into the DNA of a host cell.

The use of constructs harboring the insulator segment is envisioned for the creation of knockout mice to determine the effects of a gene on development, or for the testing of therapeutic agents, such as chemotherapeutic or other types of drugs.

In general, the constructs of the present invention contain the insulator sequence of SEQ ID NO:1, an enhancer element and a transcription unit comprising, at a minimum, a gene of interest, for example, a gene encoding a protein or precursor thereof, and a promoter to drive the transcription of the gene of interest, and other sequences necessary or required for proper gene transcription and regulation (e.g. start and stop sites, origin of replication, splice sites and polyadenylation signal). The enhancer is located in sufficient proximity to the transcription unit to enhance the transcription thereof. The constructs may contain more than one small insulator of the invention, preferably in tandem, which are positioned so as to insulate the reporter gene and its transcription unit from surrounding DNA at the site of integration.

Transcriptionally competent transcription units can be made by conventional techniques. In a preferred aspect of the present invention, the insulator element is situated between the enhancer and the promoter of a given gene to buffer the effects of a *cis*-acting DNA region on the promoter of the transcription unit. In some cases, the insulator can be placed distantly from the transcription unit. In addition, the optimal location of the insulator element can be determined by routine experimentation for any particular DNA construct. The function of the insulator element is substantially independent of its orientation, and thus the insulator can function when placed in genomic or reverse genomic orientation with respect to the transcription unit to insulate the gene from the effects of *cis*-acting DNA sequences of chromatin.

The constructs as described herein may be used in gene transfer and gene therapy methods to allow the protected expression of one or more given genes that are stably transfected into the cellular DNA. The constructs of the invention

would not only insulate a transfected gene or genes from the influences of DNA surrounding the site of integration, but would also prevent the integrated constructs from impacting on the DNA at the site of integration and would therefore prevent activation of the transcription of genes that are harmful or detrimental to the cell.

The specificity of the constructs of the invention involves transfecting the particular gene(s) of interest into a cell type having the appropriate milieu for transcription of the gene(s) whose products are desired to be expressed. The constructs of the invention are capable of being transfected into a variety of cell and tissue types. In addition, since the insulator element itself is not cell or tissue specific, it is a universal element which can act as a part of the constructs of the invention to insulation gene expression in the absence of strict cell or tissue specificity. The constructs can be designed to contain the appropriate regulatory sequences and all of the necessary DNA elements for integration of the construct and/or the appropriate components thereof and expression of a gene of interest in a given cell type.

For assembly of the construct, the insulator element for ligation can be positioned in accordance with the desired use of the constructs of the invention. Thus, as disclosed above, at least one insulator may be positioned between an enhancer element and a promoter in a transcription unit, or the insulator can be otherwise positioned on either side of a gene so as to obtain optimal insulation of the gene or genes desired to be transcribed. The insulator element can be obtained from natural sources or by synthetic means. For example, the insulator element can be excised from genomic or cDNA clones of eukaryotes, including chickens, mice, and humans, and the like, and then ligated with segments of DNA comprising the enhancer and the transcription unit. Alternatively, the insulator element can be synthetically produced by conventional techniques of DNA synthesis such as the phosphite triester chemistry method (for example, see U.S. Patent No. 4,415,732 to Caruthers et al.; and Sinha, N.D. et al., 1984).

Those skilled in the art will appreciate that a variety of enhancers, promoters, and genes are suitable for use in the constructs of the invention, and that the constructs will contain the necessary start, termination, and control sequences for proper transcription and processing of the gene of interest when the construct is

introduced into a vertebrate cell, such as that of mammal or a higher eukaryote. The constructs may be introduced into cells by a variety of gene transfer methods known to those skilled in the art, for example, gene transfection, lipofection, microinjection, electroporation, transduction and infection. In addition, it is envisioned that the invention can encompass all or a portion of a viral sequence-containing vector, such as those described in U.S. Patent No. 5,112,767 to P. Roy-Burman and D.A. Spodick, for targeted delivery of genes to specific tissues. It is preferred that the constructs of the invention integrate stably into the genome of specific and targeted cell types.

Further, the DNA construct comprising the insulator element, enhancer and transcription unit may be inserted into or assembled within a vector such as a plasmid or virus, as mentioned above. The construct can be assembled or spliced into any suitable vector or cosmid for incorporation into the host cell of interest. The vectors may contain a bacterial origin of replication so that they can be amplified in a bacterial host. The vectors may also contain, in addition to a selectable marker for selection of transfected cells, as in the exemplary constructs, another expressible and selectable or marker gene of interest.

Vectors can be constructed which have the insulator element in appropriate relation to an insertion region for receiving DNA encoding a protein or precursor thereof. The insertion region can contain at least one restriction enzyme recognition site.

A particularly useful vector for gene therapy is the retroviral vector. A recombinant retroviral vector may contain the following parts: an intact 5′ LTR from an appropriate retrovirus, such as MMTV, followed by DNA containing the retroviral packaging signal sequence; the insulator element placed between an enhancer and the promoter of a transcription unit containing the gene to be introduced into a specific cell for replacement gene therapy; a selectable gene as described below; and a 3′ LTR which contains a deletion in the viral enhancer region, or deletions in both the viral enhancer and promoter regions. The selectable gene may or may not have a 5′ promoter that is active in the packaging cell line, as well as in the transfected cell.

The recombinant retroviral vector DNA can be transfected into the amphotrophic packaging cell line Ψ-AM (see Cone, R. and Mulligan, R., 1984) or other packaging cell lines which are capable of producing high titer stocks of helper-free recombinant retroviruses. After transfection, the packaging cell line is selected for resistance to G418, present at appropriate concentration in the growth medium.

Adenoviral vectors (e.g. DNA virus vectors), particularly replication-defective adenovirus vectors, or adeno-associated vectors, are also suitable for use and have been described in the art (S. Kochanek et al., 1996; G. Ascadi et al., 1994; Ali et al., 1994).

Examples of transfectable reporter or heterologous genes that can be used in the present invention include those genes whose function is desired or needed to be expressed *in vivo* or *in vitro* in a given cell or tissue type. Genes having significance for genetic or acquired disorders are particularly appropriate for use in the constructs and methods of the invention. Genes that may be insulated from *cis*-acting regulatory sequences by the insulator elements of the present invention may be selected from, but are not limited to, both structural and non-structural genes, or subunits thereof. Examples include genes which encode proteins and glycoproteins (e.g. factors, cytokines, lymphokines), enzymes (e.g. key enzymes in biosynthetic pathways), hormones, which perform normal physiological, biochemical, and biosynthetic functions in cells and tissues. Other useable genes are selectable antibiotic resistance genes (e.g. the neomycin phosphotransferase gene (Neo ®) or the methotrexate-resistant dihydrofolate reductase (dhfr) gene) or drug resistance genes (e.g. the multi-drug resistance (MDR) genes), and the like. Further, the genes may encode a precursor of a particular protein, or the like, which is modified intracellularly after translation to yield the molecule of interest. Further examples of genes to be used in the invention may include, but are not limited to, erythroid cell-specific genes, B-lymphocyte-specific genes, T-lymphocyte-specific genes, adenosine deaminase (ADA)-encoding genes, blood clotting factor-encoding genes, ion and transport channel-encoding genes, growth factor receptor- and hormone receptor-encoding genes, growth factor- and hormone-encoding genes, insulin-encoding genes, transcription factor-encoding genes, protooncogenes, cell

cycle-regulating genes, nuclear and cytoplasmic structure-encoding genes, and enzyme-encoding genes.

The present invention is also applicable to targeting tumor or malignant cells with the insulator element-containing constructs carrying genes encoding toxins or toxoids, e.g. diphteria toxoid and the like, to kill or otherwise damage and destroy the targeted cells. In addition, newly-cloned and isolated genes may be suitable candidates for use as reporter genes in the present invention.

Examples of eukaryotic promoters suitable for use in the invention are may include, but are not limited to, the thymidine kinase (TK) promoter, the alpha globin, beta globin, and gamma globin promoters, the human or mouse metallothionein promoter, the SV40 promoter, retroviral promoters, cytomegalovirus (CMV) promoter, and the like. The promoter normally associated with a particular structural gene which encodes the protein of interest is often desirable, but is not mandatory. Accordingly, promoters may be autologous (homologous) or heterologous. Suitable promoters may be inducible, allowing induction of the expression of a gene upon addition of the appropriate inducer, or they may be non-inducible.

Further, a variety of eukaryotic enhancer elements may be used in the constructs of the invention. Like the promoters, the enhancer elements may be autologous or heterologous. Examples of suitable enhancers include, but are not limited to, erythroid-specific enhancers, (e.g. as described by Tuan, D. et al., and in U.S. Patent No. 5,126,260 to I.M. London et al.), the immunoglobulin enhancer, virus-specific enhancers, e.g. SV40 enhancers, or viral LTRs, pancreatic-specific enhancers, muscle-specific enhancers, fat cell-specific enhancers, liver specific enhancers, and neuron-specific enhancers.

Many types of cells and cell lines (e.g. primary cell lines or established cell lines) and tissues are capable of being stably transfected by or receiving the constructs of the invention. Examples of cells that may be used include, but are not limited to, stem cells, B lymphocytes, T lymphocytes, macrophages, other white blood lymphocytes (e.g. myelocytes, macrophages, monocytes), immune system cells of different developmental stages, erythroid lineage cells, pancreatic cells, lung cells, muscle cells, liver cells, fat cells, neuronal

cells, glial cells, other brain cells, transformed cells of various cell lineages corresponding to normal cell counterparts (e.g. K562, HEL, HL60, and MEL cells), and established or otherwise transformed cells lines derived from all of the foregoing. In addition, the constructs of the present invention may be transferred by various means directly into tissues, where they would stably integrate into the cells comprising the tissues. Further, the constructs containing the insulator elements of the invention can be introduced into primary cells at various stages of development, including the embryonic and fetal stages, so as to effect gene therapy at early stages of development.

In another embodiment of the invention, the constructs may be designed to contain genes encoding two subunits or components of a single protein so that each chain could be expressed from the same plasmid or suitable vector. For example, some proteins such as growth factors, growth factor receptors, blood clotting factors, and hormones are frequently comprised of two chains or subunits (e.g. α and ß) which associate to form the functional molecule. In this embodiment, the gene coding for one chain or subunit of the molecule can be positioned in the plasmid or vector in conjunction with the insulator elements and specific promoter and enhancer elements (or heterologous promoter and enhancer, if desired), and the gene coding for the other chain or subunit can be positioned in the same plasmid or vector in conjunction with its insulator, promoter, and enhancer elements. The plasmid or vector containing the dual chain-encoding genes with their appropriately-positioned insulator elements can be transfected into cells to allow for the expression of a complete, two-chained molecule from the incorporated plasmid DNA, with each chain being regulated independently and with the copy numbers remaining the same.

When used in gene transfer and gene therapy, the constructs described herein may be administered in the form of a pharmaceutical preparation or composition containing a pharmaceutically acceptable carrier, diluent, or a physiological excipient, in which preparation the vector may be a viral vector construct, or the like, to target the cells, tissues, or organs of interest. The composition may be formed by dispersing the components in a suitable pharmaceutically-acceptable liquid or solution such as sterile physiological saline or

other injectable aqueous liquids. The composition may be administered parenterally, including subcutaneous, intravenous, intramuscular, or intrasternal routes of injection. Also contemplated are intranasal, peritoneal or intradermal routes of administration. For injectable administration, the composition is in sterile solution or suspension or may be emulsified in pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives, stabilizers, and material for rendering the solution or suspension isotonic with body fluids (i.e. blood) of the recipient. Excipients suitable for use are water, phosphate buffered saline, pH 7.4, 0.15 M aqueous sodium chloride solution, dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof. The amounts or quantities, as well as routes of administration, used are determined on an individual basis, and correspond to the amounts used in similar types of applications or indications known to those of skill in the art.

Also contemplated by the invention is a kit or kits containing insulator constructs in which the insulator elements of the invention are provided in a DNA receivable vector or plasmid that contains or can be readily adapted by the user to contain the appropriate DNA elements for proper expression of a gene or genes of interest. The insulator element-containing plasmids or vectors of the kit contain insulator elements, enhancers, a transcription unit, and the gene or genes of interest may be inserted downstream of the insulator(s), as desired. Alternatively, the constructs of the kit may contain some or all of the necessary genetic elements for proper gene expression, or combinations of these, and the remaining genetic elements may be provided and readily inserted by the user, preferably between the insulator elements in the construct. The insulator element-containing plasmids or vectors may be provided in containers (e.g. sealable test tubes and the like) in the kit and are provided in the appropriate storage buffer or medium for use and for stable, long-term storage. The medium may contain stablizers and may require dilution by the user. Further, the constructs may be provided in a freeze-dried form and may require reconstitution in the appropriate buffer or medium prior to use.

The present invention further provides an insulator element found in the insulin-like growth factor 2 (Igf 2) locus. The insulator element contains a set of CTCF binding sites and comprises the sequence shown in SEQ ID NOS:84-87

(mouse), SEQ ID NOS:88-91 (rat), or SEQ ID NOS:92-98 (human). The Igf2 gene encodes a growth promoting (or mitogenic) protein and the expression of this gene and its neighboring gene H19 are imprinted. Expression of *Igf2* occurs exclusively from the paternally inherited allele. The insulator element identified is within a region between the *Igf2* and *H19* genes that is methylated in the paternal allele only.

According to the present invention, the enhancer-blocking activity of the insulator element identified in the Igf2 locus is dependent upon CTCF binding to the insulator. Methylation of the insulator element (cytosines in the sequence that are followed by a guanine (CpG) are methylated) abolishes the ability of CTCF to bind to the insulator and would result in loss of CTCF-dependent enhancer-blocking activity. Therefore, on the paternal allele, methylation of the insulator element prevents CTCF binding to the insulator element which results in Igf2 expression, whereas in the maternal allele where the insulator element is unmethylated, CTCF is capable of binding to the insulator element and prevent Igf2 expression.

As methylation is likely to be the general mechanism by which an insulator whose enhancer-blocking activity is dependent upon CTCF binding is regulated, the invention also provides methods of modulating the CTCF-dependent enhancer-blocking activity of an insulator element by targeted methylation and demethylation of the insulator element. The gene of interest may be introduced into cells by employing constructs or vectors in which the insulator element is strategically positioned with respect to the gene of interest, the promoter and the enhancer element so as to regulate the expression of the gene as described above. In one embodiment, a methylase is employed to methylate the insulator element, thereby activating the expression of the gene of interest in the cell. In one method, DNA methyltransferase 3, which has been shown to be capable of de novo methylation of the cytosine of the CpG residues both in vivo and in vitro (See Bird, 1999 for review), is employed. In this method, in addition to the gene of interest, the promoter, the enhancer element and the insulator element, the vector introduced into the cells also comprises a DNA binding sequence of a DNA binding protein, for example, Gal4 or LexA. The DNA binding sequence would be located in regions adjacent to the CpG residues to be methylated in the insulator element. A vector encoding a fusion protein in which the enzymatic domain of the DNA

methyltransferase 3 is fused to the DNA binding domain of the DNA binding protein is then introduced into the cells. Methods for generating such domain-fusion proteins are well known to those skilled in the art. Binding of the fusion protein to the DNA binding sequence adjacent to the CpG residues and the expression of the fusion protein allow the CpG residues to be methylated by the methyltransferase. In another embodiment, a similar method may be employed to prevent the expression of the gene of interest in a cell by demethylating the CpG residues using a demethylase.

Examples

The examples as set forth herein are meant to exemplify the various aspects of carrying out the invention and are not intended to limit the invention in any way.

Example 1

Materials and Methods

A. Plasmid Construction and Oligonucleotides

The plasmid pNI was the base plasmid in which all DNA fragments were tested for enhancer blocking activity.

DNA fragments subcloned into the AscI site of this plasmid are located between the enhancer (mouse HS2) and the reporter ("γ-neo"). When cloned into the NdeI site of pNI, inserted DNA sequences are located "upstream" of the enhancer, and when cloned into the XbaI site, the sequences are located "downstream" of the reporter; in both of these cases the insert is located outside the promoter-enhancer path.

pNI was generated by replacing the SacI copy of the 1.2 kb insulator (found between the enhancer and the promoter) in pJC5-4 (Chung et al, 1993) with an AscI linker (New England Biolabs) after digestion of this plasmid with EcII36II. The following primers were used in PCR amplifications to generate fragments for cloning into the AscI site of pNI:

AC1F (SEQ ID NO:29); AC1R (SEQ ID NO:30); AC2F (SEQ ID NO:31); AC2R (SEQ ID NO:32); ΔIACF (SEQ ID NO:33); ΔIiacF (SEQ ID NO:34); ΔIiacR (SEQ ID NO:35); ΔIIIACF (SEQ ID NO:36); ΔIIIACR (SEQ ID

NO:37); ΔIVACF (SEQ ID NO:38); ΔIVACR (SEQ ID NO:39); ΔVACR (SEQ ID NO:40); BEADascF (SEQ ID NO:41); BEADascR (SEQ ID NO:42); BEADΔAF (SEQ ID NO:43); and BEADΔAR (SEQ ID NO:44).

The core, FI/FII, FIII/FIV/FV, ΔI and ΔV were generated by PCR using the plasmid p50I (Reitman and Felsenfeld, 1990) as a template and the primer pairs AC1F/AC2R, AC1F/AC1R, AC2F/AC2R, ΔIACF/AC2R, and AC1F/ΔVACR, respectively. Deletions of FII, FIII and FIV from the core were accomplished by two-step, overlapping PCR. For each deletion, a pair of intermediate fragments was generated by PCR in separate reactions using p501 as the template and the primer pairs AC1F/ΔIIacR, ΔIIacF/AC2R, AC1F/ΔIIIACR, ΔIIIACF/AC2R, AC1F/ΔIVACR, and ΔIVACF/AC2R. The products of each of these reactions were gel-purified, mixed pair-wise to generate the appropriate templates, and the final products were amplified with AC1F/AC2R (for example, the products of amplifications with AC1F/ΔIIacR and ΔIIacF/AC2R were mixed to generate the template for PCR of the core-ΔII).

The full-length BEAD-1 fragment was generated by PCR from K562 genomic DNA with primers BEADascF and BEADascR. The fragment BEADΔA was generated in a two-step, overlapping PCR reaction, first using the BEAD-1 fragment as a template and the primers BEADascF/BEADΔAR and BEADΔAF/ BEADascR in separate reactions, then mixing the gel-purified products of these reactions with primers BEADascF and BEADascR to generate the final product by PCR.

The a 1.6 kb fragment containing the full length RO element was subcloned into Ec1136II cut pJC5-4 after liberation of this fragment from p0, 1 (Robinett et al., 1997) by digestion with Ec1136II and PvuII. All other enhancer-blocking fragments were generated by direct synthesis of the appropriate complementary oligonucleotides on an ABI 394 DNA synthesizer. The top strands of these were FII/FIII: (SEQ ID NO:45); FII/III-ΔSp1* (SEQ ID NO:46); FII/III-Δα2 (SEQ ID NO:47); Δ spacer (SEQ ID NO:48); ΔIIN (SEQ ID NO:49); ΔIIIN (SEQ ID NO:50); FII (SEQ ID NO:51); FIII (SEQ ID NO:52); gypsy-3 (SEQ ID

NO:53); mycFV (SEQ ID NO:54); lys (SEQ ID NO:55); ApB (SEQ ID NO:56); RO100 (SEQ ID NO:57); and BEAD-A (SEQ ID NO:58).

All FII mutants were identical to FII, except for those bases indicated in lowercase in Fig. 3D. For use in the enhancer-blocking assay, single stranded oligonucleotides were purified by denaturing PAGE, quantified, annealed, digested with AscI, and subcloned into pNI. The FII site was also generated as above with NdeI sites at its ends for cloning upstream of the enhancer in pNI to generate FII-UP. To generate FII-DOWN, FII was digested out of pNI-FII, the ends were flushed with Klenow, and XbaI linkers (New England Biolabs) were added for cloning into the XbaI site of pNI.

The 2.5 kb Imprinted Control Region (ICR) fragment and the 1.6 kb deleted fragment (DMD) within the ICR were generated by PCR on genomic DNA with ICRR (SEQ ID NO:62) and ICRF (SEQ ID NO:63); ICRR and DMDF (SEQ ID NO:64) primers, respectively. The ~800 bp HS1 fragment was generated with ICRF and HS1R (SEQ ID NO:65); and the HS2 fragment was generated with HS2F (SEQ ID NO:66) and ICRR. Deletions of m1 and m2 from HS1 were accomplished by PCR using the following additional primers: HS1Δm1F (SEQ ID NO:67); HS1Δm1R (SEQ ID NO:68); HS1Δm2F (SEQ ID NO:69); HS1Δm2R (SEQ ID NO:70); HS2Tm4R (SEQ ID NO:71); HS2F (SEQ ID NO:72); HS2Tm3F (SEQ ID NO:73); HS2Δm3F (SEQ ID NO:74); HS2Δm3R (SEQ ID NO:75). The fragments Δm3 and Δm4 are ~200 base pair truncations of the 5' and 3' of HS2 generated by PCR with the primer pairs HS2Tm3F/ICRR and HS2F/HS2Tm4R, respectively. In the fragment Δm3Δm4, ~90 base pairs spanning the m3 site were internally deleted while the deletion of m4 results from a 3'-truncation. This was accomplished by two-step overlapping PCR using the primer pairs HSΔm3F/HS2Tm4R and HS2F/HS2Δm3R on a DMD clone template. The products of these reactions were gel-purified, mixed, and the final product was amplified by PCR with the primer pairs HS2F/HS2Tm4R. Internal deletions of ~90 bp fragments spanning m1 and m2 from HS1 were generated by first amplifying with the primer pairs HS1F/HS1Δm1R, HS1Δm1F/ HS1R, HS1Δm1F/HS1Δm2R, HS1F/HS1Δm2R, and HS1Δm2F/HS1R. To generate singly or doubly deleted fragments the products of

these reactions were gel-purified, mixed accordingly, and amplified with HS1F/HS1R. The resulting fragments were sub-cloned into pNI after addition of the appropriate linkers where necessary. For enhancer blocking assays with m3, 5'-GCTGTTATGTGCAACAAGGGAACGGATGCTACCGCGCGGTGGCAGCATA CTCCTATATATCGTGGCCCAAATGCTGCCAACTTGGGGGGAGCGATTCA TTC (SEQ ID NO:83) was directly synthesized with the appropriate restriction sites at its ends and cloned into pNI at either the AscI or NdeI sites.

B. Enhancer Blocking Assay

Enhancer blocking assays were performed as previously described (Chung et al., 1993 and Chung et al., 1997). Briefly, 20 µg of each construct was linearized by SalI digestion, phenol-chloroform extracted, ethanol precipitated, and quantified by UV absorption. 20 ng of each DNA was then electroporated into K562 cells (1×10^7) and after allowing 24 hours for recovery, cells were plated in soft agar with geneticin (Life Technologies) at 750 µg/ml (active). Colonies were counted after 3 weeks of selection and the colony number was normalized to that obtained with pNI or a construct which had 2.3 kb of λ DNA inserted between the enhancer and the reporter as a spacer control.

C. DNase I Hypersensitive Site Analysis

Nuclei were isolated from adult chicken red blood cells, essentially as described previously (Bresnick and Felsenfeld, 1994), except that 0.2 mM EGTA was included in all buffers. After incubation of the nuclei with varying concentrations of DNase I for 5 minutes at room temperature, the reaction was terminated by the addition of SDS and the genomic DNA was purified. To map precisely the position of HS4, DNase I digested and undigested genomic DNAs (10 µg) were further digested with StyI to generate an ~I kb parent fragment which spanned the insulator core. StyI digested control DNAs were also digested with the enzymes indicated in Fig. 1A. All of these DNAs were resolved on a 1.3 % agarose gel and subjected to Southern blotting by standard techniques using a 503 bp StyI-SacI fragment from the plasmid p501 as a probe (Reitman and Felsenfeld, 1990).

D. DNA Binding Assays

All DNA binding assays were carried out in a binding buffer containing 20 mM HEPES, pH 7.9; 150 mM KCl; 5 mM $MgCl_2$; and 1 mM DTT. For gel mobility shift assays, DNA binding was carried out at room temperature for 30 minutes in binding buffer plus 5% glycerol, 20-40 fmol of labeled double stranded oligonucleotide probe, poly dI/dC at 50-I00 μg/ml, and 1-5 μl of protein in a final volume of 20 μl. Probes were oligonucleotide duplexes identical to those used for subcloning into the enhancer-blocking assay. 10 pmol of each top strand was end-labeled with ^{32}P and then annealed with 15 pmol of an unlabeled complementary oligonucleotide; the resulting duplexes were used directly as probes. Cold competitor duplexes were added simultaneously with labeled probes at 50-fold molar excess.

An antibody was raised against a C-terminal peptide (APNGDLTPEMILSMMD), SEQ ID NO: 59, of CTCF. Supershifts were carried out by pre-incubating the appropriate proteins with purified antibodies in binding buffer for 2 hours at 0° C, followed by a room temperature incubation of 30 minutes in the presence of DNA.

For southwestern assays, proteins were resolved by SDS-PAGE, transferred to PVDF, and then denatured and renatured by successive 10 minute incubations in binding buffer supplemented with guanidine hydrochloride at 4.8, 3, 1.5, and 0.75 M. After an additional 10 minute wash in binding buffer, the blots were blocked in binding buffer plus 5% non-fat dry milk for 16 hours at 4° C. An FII probe was generated for southwestern assays by annealing a full length top strand sequence: (CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGG-CAGCAGGCGCGCCT), (SEQ ID NO:60) to a short 3' complementary primer (AGGCGCGCCTGCTGC), (SEQ ID NO:61). This partial duplex was then extended with Klenow in the presence of α-^{32}P-dCTP resulting in a probe identical to that used in gel-shift assays but with 10 labeled phosphates per molecule. Blots were probed for 3 hours at room temperature in binding buffer supplemented with 0.25% non-fat dry milk, 5 μg/ml poly dI/dC and 3 pmol of labeled probe in a final volume of 20 ml, washed three times for 10 minutes in the same buffer without DNA and exposed to film.

Probes used in the DNA binding assays described in Example 6 were annealed duplexes of the following sequences: m1 (SEQ ID NO:76); m2 (SEQ ID NO:77); m3 (SEQ ID NO:78); m4 (SEQ ID NO:79); h1 (SEQ ID NO:80); FIIx3' (SEQ ID NO:81); m1x3' (SEQ ID NO:82).

E. Protein Purification and Translation

Nuclear extracts from K562 cells and whole chicken blood were prepared essentially as previously described (Evans et al., 1988). For purification of the FII binding protein, nuclei were prepared from 6 liters of whole chicken blood (Pelfreez Biologicals) and extracted in Buffer C: 20 mM HEPES, pH 7.9, 420 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA and 1 mM DTT. The resulting extract was diluted to 150 mM NaCl and 20% glycerol and fractionated on a 500 ml SP sepharose column (Pharmacia) using a 0.15-1M NaCl linear gradient. Active fractions were pooled, diluted to 150 mM NaCl and loaded onto a 25 ml CM sepharose column (Pharmacia). Active fractions eluted with a peak at 600 mM NaCl from a 0.15-1M NaCl gradient. These fractions were pooled and loaded onto a 2.6/60 cm Sephacryl S-300 gel filtration column (Pharmacia) that was pre-equilibrated with 20 mM HEPES, pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA and 1 mM DTT. Active fractions were pooled, dialyzed into 10 mM sodium phosphate pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 20% glycerol, and loaded onto a 25 ml Macro-Prep ceramic hydroxyapatite column (Bio-Rad). This column was eluted with a 10-800 mM phosphate gradient at pH 8.0. Throughout the isolation all buffers were supplemented with 1 mM PMSF, 0.7 μg/ml pepstatin, and 0.5 μg/ml leupeptin and maintained at 4° C. Fractions pooled from the gel-filtration, and all subsequent buffers, were also supplemented with 40 μg/ml bestatin and AEBSF (at 200 μg/ml) was substituted for PMSF. Final active fractions were identified by gel shift assay, and analyzed by southwestern with an FII probe. For peptide sequencing, 1 ml of a final active fraction (representing ~1/10th of the final yield and ~5 μg of purified ~140 kDa protein) was TCA precipitated, resolved on 7% Tris-acetate SDS-PAGE (Novex), transferred to PVDF, stained with imido black, and internal protein sequence data were obtained at the Rockefeller University Protein/DNA Technology Center.

In vitro-translated human CTCF was obtained using the plasmid p4B7.1 as a template for in vitro transcription by T7 polymerase according to the manufacturers instructions (Ambion, 'Message Machine'), followed by in vitro translation of the resulting RNA in a nuclease treated rabbit reticulocyte system (Promega). The plasmid p4B7.1 contains the full length human CTCF cDNA (Filippova, et al., 1996) subcloned into pCITE4b(+) (Novagen).

Example 2

Identification of an Enhancer-Blocking DNA Fragment

A 1.2 kb DNA element at the 5' end of the chicken ß-globin locus, corresponding to a constitutive DNase I hypersensitive site (5'HS4), was shown to function as an insulator in an enhancer-blocking assay (U.S. Patent No. 5,610,053 to J. Chung et al.; Chung et al., 1997). The assay tested the ability of a DNA sequence to prevent activation of a gene for neomycin resistance by a strong enhancer when the construct was stably transformed into an erythroleukemia cell line (Chung et al., 1993). The insulator effect was manifested by a marked reduction in the number of colonies resistant to G418 only when the globin sequence element was placed between the enhancer and the promoter. This same assay was utilized to show that a large part of the insulator activity was contained in a 250 bp GC-rich 'core' fragment at the 5' end of the 1.2 kb element. HS4 mapped precisely within this core region, consistent with its significance *in vivo*. (Fig. 1A).

DNase I footprinting of the 250 bp core insulator sequence with nuclear extracts revealed five protected regions (FI to FV), as illustrated in Fig. 1C; Chung et al. 1997). The FI-FV DNA segment was further dissected and analyzed to identify an insulator-protein binding site. The core region was divided into separate fragments; each fragment was employed in enhancer blocking assays. Splitting the core between FII and FIII generated two fragments (FI/FII and FIII/IV/V) each of which had some enhancer blocking activity. However a fragment containing only FII and FIII had greater activity than the entire core (FII/III, Fig. 1D).

Deletion analysis confirmed that FII and FIII were responsible for the majority of the enhancer blocking activity. While deletion of FI had a slight effect, deletion of FII and FIII significantly reduced enhancer-blocking activity (Fig. 1E).

Deletions of FIV and FV were essentially neutral. Considered together, these results show that regions FII and FIII represent a functional enhancer-blocking region of the core. Consistent with this conclusion, the insertion of an increasing number of copies of FII/FIII between the enhancer and the promoter resulted in a linear increase in blocking activity, as was also observed for the 1.2 kb insulator and the 250 bp core sequence (Fig. 1F).

Further analysis of the FII/FIII element revealed an internal 'spacer' sequence that appeared to partially counteract the enhancer-blocking activity. Removal of this spacer region (Fig. 2A) resulted in even stronger blocking activity. In fact, the removal of sequences adjacent to FII resulted in the discovery of an approximately 50 bp sequence spanning FII that alone was found to possess a blocking activity nearly equal to that of the full 1.2 kb insulator element. Consistent with the behavior *in vivo*, it is noted that the position of FII is coincident with that of HS4 in nuclei (Fig. 1A). Importantly, enhancer-blocking by these minimal fragments, including FII, displayed the same position-dependence as that observed for the entire 1.2 kb insulator element. When placed either upstream of the enhancer or downstream of the promoter in the enhancer blocking assay, FII had essentially no effect on expression (Fig. 2B; Chung et al., 1997). Thus, in order to effect expression, FII must be located between the enhancer and the promoter (Fig. 2B).

Example 3

Identification of a Specific Enhancer-Blocking Protein

Experiments were conducted to identify proteins that bound to the FII fragment, in view of the strong enhancer-blocking activity exhibited by this fragment. A comparison of the sequence of FII with that of known transcription factor binding sites revealed several potentially significant homologies (Fig. 2E). An Sp1 consensus sequence lies in the middle of the FII fragment and a sequence homologous to a yeast $\alpha 2$ binding site (Sauer et al., 1988) overlaps a partial match to the binding site of the *Drosophila* protein suppressor of Hairy-wing (Su(Hw)), (Fig. 2E; Geyer and Corces, 1992). To test whether any of these homologies could account for the blocking activity of FII, mutations were introduced that were

predicted to reduce dramatically the affinity of each of these proteins for the sequence of the FII fragment. The mutations were introduced into either the FII or the FII/III fragments and were tested in the enhancer-blocking assay. A deletion of 4 base pairs within the region that overlaps both the α2 and the Su(Hw) binding sites had no effect on the blocking activity of the FII/III fragment (Fig. 2D). Furthermore a 100 bp fragment, derived from the *Drosophila gypsy* element and containing three canonical Su(Hw) binding sites had no activity in the assay. Thus, it was concluded that neither the Su(Hw) site nor the α2 site can account for the activity contained in FII.

Similarly, in the context of FII, mutation of the Sp1 consensus sequence had no effect on the blocking activity of the fragment; in fact, mutation of each of the three potential Sp1 binding sites in FII/III resulted in substantially increased activity (Fig. 2D). Sp1 may act as an inhibitor of enhancer-blocking in the enhancer-blocking assays described here. This may also explain the above-mentioned inhibitory effect of the "spacer" sequence between FII and FIII and account for the observation that the activities of FII and FIII are not always additive (see, for example, Δ spacer in Fig. 2A). In addition, mutation of the Sp1 sites in FII/III rendered the level of enhancer-blocking activity equal to the sum of that of FII and FIII.

To determine which sequences within FII were responsible for its activity, multiple transversions (e.g., C√A and G√T) were made across the 5', middle and 3' regions of the fragment (Fig. 3A). All of these transversions reduced the level of enhancer-blocking activity of FII, but changes at the 3' end of the fragment (x3') caused a complete loss of activity. In addition, deletion of 10 bp from both ends of FII (ΔF), or a reversal of the sequence 5' - 3' (rev), resulted in dramatic reductions in activity. An effect of sequence composition is ruled out by the "rev" mutant since its base composition is identical to that of FII.

In light of the foregoing, experiments were carried out to identify protein(s) that bounds to FII with a competition profile that matched the sequence specificity observed in the enhancer-blocking assay. Nuclear extracts were prepared from the human erythroleukemic cell line K562 (the cell line in which the enhancer-

blocking assay was performed) and from adult chicken red blood cells (the FII insulator is a chicken-derived element). Identical patterns were obtained with these two extracts in a gel mobility-shift assay (Figs. 3B and 3C). For each cell source of nuclear extract, two major complexes were observed when the extract was incubated with a 60 bp probe spanning footprint II. The upper complex could be super-shifted with an antibody against Sp1 and was competed by a 100-fold excess of an unlabeled oligomer carrying an Sp1 consensus binding site. This complex was concluded to contain Sp1. In contrast, the lower complex was neither super-shifted by anti-Sp1 antibody, nor was its binding influenced by an excess of Sp1 consensus binding site. Importantly, the degree to which each of the tested fragments competed for binding to this complex paralleled its ability to act as an insulator in the enhancer-blocking assay (Fig. 3D).

Example 4

Isolation of a Protein Responsible for the Sequence Specificity of Enhancer-Blocking

Probing a southwestern blot of nuclear proteins with labeled FII revealed a single FII-specific DNA binding protein with an apparent size on gels of ~140 kDa (Fig. 4A). This protein was purified by conventional chromatography. Throughout the purification, the elution profiles of FII binding activities were identical in gel-mobility shift and southwestern assays. This protein bound tightly to S, CM, and hydroxyapatite columns, and eluted with a peak at ~330 kDa on gel filtration (Fig. 4B). Coomassie staining of gels of the final hydroxyapatite fractions revealed a single protein with an apparent molecular weight of 140 kDa corresponding to the position of the FII southwestern activity (Fig. 4D). The sequences of four internal peptides (Fig. 4C) from the 140 kDa DNA binding component of the final purified fraction all perfectly matched the predicted sequence of a previously cloned 11 zinc finger DNA binding protein, CTCF (Klenova, 1993; Filippova et al., 1996).

Consistent with this identification, *in vitro*-translated CTCF bounds to FII with a sequence specificity identical to that observed in the gel-mobility shift and enhancer-blocking assays (Fig. 5). As expected, this protein also bound to other previously characterized CTCF sites (Fig. 5, lanes 9-11) and these sites also act as

enhancer blockers in our assay (Figure 5). Alignment of these CTCF sites with FII revealed a conserved region which has been shown to be critical for binding of CTCF to these other sites (Filippova et al., 1996; Burcin et al., 1997; Vostrov and Quitschke, 1997). Mutation of this conserved 3' sequence completely abrogated binding and enhancer blocking in the relevant binding and enhancer-blocking assays (see x3' in Figs. 3A-3D and alignment in Fig. 6A).

Example 5

Conservation of Sequence Among Vertebrate Insulators

Because CTCF is highly conserved among vertebrates, an investigation as to whether CTCF sites might be present in other vertebrate insulator elements was carried out. Two such elements have recently been described. A 1.4 kb fragment found in the intergenic spacer region of the ribosomal RNA genes of *Xenopus laevis*, termed the repeat organizer (RO), has been shown to prevent enhancer action in a directional manner (Robinett et al., 1997). The 3' half of this sequence is composed of seven tandem repeats of an ~100 bp GC-rich sequence (Labhart and Reeder, 1987). The RO sequences bear significant homology with CTCF sites, including FII (Fig. 6B). In the enhancer-blocking assay, the full-length RO conferred moderate enhancer-blocking activity and a single copy of the 100 bp RO repeat had weak enhancer blocking activity on its own (Fig. 7B). It is perhaps because of the weak activity of a single copy of this sequence, that attempts to obtain reproducible binding of CTCF to a single RO repeat have been unsuccessful.

The only other vertebrate insulator described to date, BEAD- 1, is a 1.6 kb enhancer-blocking element derived from the human T-cell alpha/delta (α/δ) locus (Zhong and Krangel, 1997). Best-fit alignment of this element with various CTCF sites revealed a good match between FII and a-sequence roughly at the center of this element (BEAD-A in Fig. 6C). In fact, a DNA fragment containing this region also bound specifically to purified chicken CTCF (Fig. 7A, lanes 6 and 5 respectively). Consistent with these observations, both full-length BEAD-1 and the CTCF binding BEAD-A element defined here were effective enhancer-blocking elements in the described enhancer-blocking assay (Fig. 7B). Furthermore, deletion

of the BEAD-A sequence from BEAD-1 largely eliminated the activity of the larger element.

Example 6

Methylation of CTCF Binding Sites Controls Imprinted Expression of the Igf2 gene

The gene encoding Insulin-like Growth Factor 2 (*Igf2*) and the H19 gene are neighboring genes on the same chromosome. The Igf2 gene encodes a growth promoting (or mitogenic) protein and abnormal expression of this gene has been linked to numerous cancers and is thought to play a causal role in the etiology of several growth defect-related syndromes.

Expression of the *Igf2* and *H19* genes is imprinted. Genomic imprinting refers to a mechanism through which expression of a particular gene is dependent upon the gamete (or parent) of origin. For example, a gene is expressed when on the chromosome contributed by the mother, but not expressed from the paternal chromosome. As the alleles are identical in sequence, the signal for such a mechanism cannot rely on DNA sequence. Gamete specific modification of the DNA though DNA methylation is believed to play a major role in specifying the allele's parent-of-origin.

Although the *Igf2* and *H19* genes share an enhancer (Yoo-Warren, 1988), *H19* is only expressed from the maternal allele, while expression of *Igf2* occurs exclusively from the paternally inherited allele (Bartolomer, 1991; Dechiara, 1991). A region located upstream of the mouse *H19* gene and between the *Igf2* and *H19* genes is methylated in the paternal allele only. This differentially methylated region appears to be the site of an epigenetic mark that is required for the imprinting of these genes. One study has shown that a deletion within this region results in loss of imprinting of both *H19* and *Igf2* and maternal transmission of a 1.6 kb deletion within this region results in expression of the normally silent *Igf2* allele (Thorvaldsen, 1998). The ability of this deleted fragment (DMD) to act as a positional enhancer-blocking element was examined by inserting it at various locations relative to an enhancer as shown in Figure 8. Insertion of the 1.6 kb DMD fragment between the enhancer and the promoter results in an 8–10-fold drop in colony number, similar to the 8-fold drop observed with the previously

characterized 1.2 kb chicken β-*globin* insulator (U.S. Patent No. 5,610,053). These results cannot be explained by the increased distance between the enhancer and the promoter, as insertion of up to 2.3 kb of heterologous DNA between them has little effect on colony number (U.S. Patent No. 5,610,053). Furthermore, like the β-*globin* insulator, when the DMD is placed outside the enhancer-promoter path, either upstream of the enhancer or downstream of the promoter, it has little effect on expression (Fig. 8b). Therefore, DMD appears to have the position-dependent enhancer-blocking properties of an insulator.

The DMD fragment is part of a slightly larger (~2 kb) imprinted control region (ICR) that is methylated throughout development exclusively on the paternal allele (Tremblay, 1995 and 1997). Allele-specific alterations in chromatin structure were also observed in this region (Szabo, 1998; Khosla, 1999 and Hark, 1998). Two nuclease-hypersensitive regions are located exclusively on the maternal allele (HS1 and HS2 in Fig. 8a), whereas the chromatin on the paternally derived allele is methylated and nuclease insensitive (Hark, 1998). Both HS1 and HS2 remain hypersensitive throughout development and are present independent of tissue type. The enhancer-blocking potential of fragments spanning HS1, HS2, and a larger fragment that spans the entire ICR was tested. All of these fragments confer enhancer-blocking activity (Fig. 8b). HS1 and HS2 individually show considerable enhancer-blocking activity; a fragment that contains both HS1 and HS2 essentially eliminates the enhancer's influence on expression (Fig. 8b, compare NI E with ICR).

A BestFit comparison between the FII fragment of β-globin and the 2.6 kb of sequence spanning the ICR revealed a 13/16 match between the 3'-end of FII and a sequence at the 5'-edge of HS2 (m3 in Fig. 9a). By searching the remainder of the ICR with the m3 sequence, a total of four homologous sequences (m1-4 in Fig 9a) was identified. Consistent with their *in vivo* significance, sequences homologous to these mouse sites are also found upstream of the human and rat *H19* genes (aligned in Fig. 9a); conservation of sequences overlapping those shown here was recently noted (Frevel, 1999a; Stadnick, 1999). In humans, a region of paternal-specific DNA methylation upstream of *H19* has also been defined (Jinno, 1996; Frevel, 1999b) and these homologous sequences are part of a larger

repeating element found in that region. An alignment of all of the sites from rat, human, and mouse reveals a 12 base pair consensus sequence that is shared among them (Fig. 9a). This consensus bears an 11/12 match with the sequence at the 3' of *β-globin* FII. In addition, each of these mouse sites, and a representative human site bind to both purified chicken CTCF (P) and *in vitro* translated human CTCF (I) (Figure 9c). Like FII, a fragment spanning a single mouse ICR site confers position-dependent enhancer blocking activity *in vivo* (Fig. 9b). Furthermore, in gel shifts with K562 nuclear extracts (E) (the cells in which the enhancer blocking assays were performed) a complex that comigrates with the FII/CTCF complex was observed with each of these mouse and human ICR sites (Figure 9c) and an antibody raised against CTCF supershifts this complex (Fig. 10d). Consistent with the enhancer blocking activity of CTCF, this complex was competed by FII, but not by a mutant of FII in which both enhancer blocking and CTCF binding have been eliminated (FIIx3' in Fig. 9d). When the base pairs shared among the ICR sites and FII are altered in the context of one of these mouse sites it no longer competes for binding to CTCF (m1x3' in Fig. 9d).

In the mouse *H19* ICR, HS1 and HS2 each contain two CTCF sites. The two sites were deleted sequentially and the enhancer blocking activities of the resulting fragments were measured (Fig. 10). Since the enhancer blocking activities of HS1 and HS2 are somewhat dependent upon their orientations (data not shown), deletion analyses were carried out with the orientation that gave the strongest activities. In each case, a deletion that eliminates either one of the CTCF sites results in a reduction in enhancer blocking activity, while deletion of both sites from either HS1 or HS2 eliminates their activity (Fig. 10a). The deletions span sequence that are larger than the average ~53 bp CTCF footprint. Among these sequences, however, the only significant similarity is within the CTCF sites. These similarities define a consensus for CTCF binding (Fig. 9a and d) which is essential to the enhancer blocking activity of β-globin FII. As shown in Fig. 9b, single CTCF site from several other loci (including the mouse ICR0 alone confer enhancer blocking activity.

The above results demonstrate that sequences within the mouse H19, ICR have the enhancer blocking properties of an insulator. Several recent studies

suggest that this activity is directly involved in the regulation of *Igf2*. One study showed that if the *H19* enhancer is moved from its genomic location downstream of *H19* to a new location upstream of the ICR, the normally silent maternal allele of *Igf2* is expressed (Webber, 1998). This suggests that it is the enhancer's position downstream of the *H19* locus that prevents activation of the maternal *Igf2* allele. Competition between the *H19* and *Igf2* promoters cannot explain this result since deletion of the *H19* promoter has no effect on *Igf2* expression (Schmidt, 1999). Instead, it is the enhancer's position relative to the ICR that restricts its action: a deletion within the ICR results in biallelic expression of *Igf2* (Thorvaldsen, 1998). This line of reasoning is further supported by the observation that maternal inheritance of the relocated enhancer results in loss of expression of the normally active *H19* allele, in this case because the ICR, now located between the enhancer and the *H19* promoter, blocks their interaction. Thus, the dependence of *H19* and *Igf2* expression on the position of the *H19* enhancer is explained by a single model that posits the existence of an insulator within the ICR (Thorvaldsen, 1999; Webber, 1998; Leighton, 1995).

As the *H19* locus contains an insulator that is active only on the unmethylated (maternal) allele, a model has been proposed which suggests that the influence of the ICR on expression of *Igf2* depends upon the allele's parent of origin (Leighton, 1995). In this model, inheritance of paternal-specific CpG methylation in the ICR results in inactivation of the insulator and thus on this allele the *H19* enhancer is free to activate *Igf2*. Direct support for a role of DNA methylation in activation of *Igf2* comes from the observation that in DNA methyltransferase-1 deficient mouse embryos, both alleles of *Igf2* are silent (Li, 1993).

The results of the deletion analysis of HS1 and HS2 imply that it is the conserved CTCF sites in these elements that are responsible for their enhancer blocking activities. One model that could explain why CpG methylation abolishes this activity is that CTCF cannot bind these sites when they are methylated. To test this, the corresponding oligomers were synthesized with ^{5me}C incorporated at each CpG and the ability of the resulting duplex to compete for the binding of CTCF to the unmethylated form was assessed. Methylation of each of the mouse sites, and a representative human site, greatly reduces their ability to compete for binding of

CTCF to an unmethylated site, even at a 50-fold molar excess (Fig. 10b). Methylation of β-globin FII has a similar effect. Because FII and the ICR sites have only one CpG in common, the influence of methylation at only this site (on both strands) in several ICR sites was examined (Fig. 10b, right panel). In fact, methylation of this CpG alone significantly reduced CTCF binding to all of these sites (Fig. 10b, M1 lanes). This result implies that enhancer access could, in principle, be regulated by a single (perhaps targeted) methylation event. Further examination of the influence of methylation on gene expression will require a system that allows for the establishment and maintenance of partially methylated transgenes *in vivo*.

The above results demonstrate that the *H19* ICR is an enhancer-blocking element. CTCF binding sites are required for this activity and when these sites are methylated, they no longer bind the insulator protein CTCF. These results provide direct evidence for a mechanistic explanation of Igf2 imprinting in which differential methylation of an enhancer boundary allows for epigenetic control of *Igf2* expression in the embryo (Fig. 11). In humans, a causal link between overexpression of *Igf2* and the pathogenesis of some cases of Beckwith-Wiedemann syndrome (BWS) has been suggested (Eggenschwiler, 1997; Sun, 1997; Weksberg, 1993; Joyce, 1997). BWS, or fetal overgrowth syndrome, is a disorder of prenatal overgrowth and predisposition to embryonal malignancies such as Wilms tumor. Studies have shown a correlation between loss of imprinting of *Igf2* in Wilms tumor and BWS and increased methylation of the maternal *H19* allele (Steenman, 1994; Okamoto, 1997; Reik, 1995; Taniguchi, 1995). In Wilms tumor, this aberrant methylation pattern was recently shown to include the CTCF sites illustrated in Figure 9a (Frevel, 1999b). These sites were consistently methylated on both alleles in Wilms tumors with loss of *Igf2* imprinting. The results described herein are consistent with the notion that the loss of *Igf2* imprinting observed in those tumors is caused by inactivation of a CTCF dependent insulator in that locus.

Recent evidence shows that in *Drosophila*, the activity of an insulator can be modulated by adjacent *cis*-acting sequences (Zhou, 1999). The results described herein reveal that in vertebrates the activity of enhancer boundaries can be controlled by DNA methylation. Not relegated simply to the role of a fixed

boundary, some insulators may act as switches that provide a novel kind of modulated gene regulation.

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All of the references cited herein above are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated DNA molecule comprising SEQ ID NO:1, said DNA molecule having enhancer-blocking function.

2. An expression construct comprising the DNA molecule of claim 1.

3. The construct according to claim 2, wherein the construct is operable when inserted into the DNA of a cell to insulate the expression of one or more genes from one or more *cis*-acting regulatory sequences in chromatin.

4. The construct according to claim 3, wherein said cell is a mammalian cell.

5. A vector construct comprising:

(a) the DNA molecule according to claim 1;

(b) a promoter domain;

(c) a gene operably linked to the promoter domain; and

(d) an enhancer domain 5′ of the promoter domain, wherein the insulator DNA molecule is positioned between the enhancer and the promoter domains so as to operably insulate the transcription and expression of the gene from *cis*-acting regulatory elements in chromatin.

6. An isolated DNA construct for incorporation into a host cell and for insulation of the expression of a gene therein, comprising:

a) DNA comprising a transcription unit comprising an expressible gene, a promoter to drive the transcription of the gene, and an enhancer element; and

b) one or more DNA molecules according to claim 1, the molecules being positioned in sufficient proximity to the transcription unit and to the gene to insulate the transcription and expression of the gene from *cis*-acting DNA regulatory sequences in chromatin outside of the DNA according to a).

7. The DNA construct according to claim 6, wherein the expressible gene is a structural gene.

8. The DNA construct according to claim 6, wherein the expressible gene is selected from the group consisting of protein-encoding genes, hormone-encoding genes, peptide hormone-encoding genes, enzyme-encoding genes, and antibiotic-resistance-encoding genes.

9. The DNA construct according to claim 8, wherein the expressible gene is a neomycin-resistance gene or a hygromycin-resistance gene.

10. A mammalian cell stably tranfected with the construct according to claim 2.

11. A method of insulating the expression of an introduced gene from cis-acting DNA regulatory sequences in the chromatin into which the gene has integrated, comprising:

a) introducing into a cell the DNA construct according to claim 2;

b) integrating the construct into the chromatin of the cell, wherein the expression of a resultant integrated heterologous gene is insulated from cis-acting DNA regulatory sequences in the chromatin of said cell.

12. The method according to claim 11, further comprising introducing into the cell a DNA construct containing a gene encoding the CTCF protein, wherein CTCF is expressed in the cell.

13. A kit for insulating the expression of a transfected and expressed gene, comprising a vector comprising the insulator molecule according to claim 1.

14. A pharmaceutical composition comprising the construct according to claim 2 in a pharmaceutically acceptable diluent, carrier, or excipient.

15. A method of blocking activity of an enhancer of a gene in a cell, comprising:

a) introducing into the cell a construct containing a DNA molecule comprising SEQ ID NO:1;

b) introducing into the cell a construct containing a gene encoding the CTCF protein, wherein the CTCF protein is expressed in the cell and binds to the DNA molecule of step (a).

16. An isolated DNA molecule comprising the sequences shown in SEQ ID NOS:84-87, said DNA molecule having enhancer-blocking function.

17. An isolated DNA molecule comprising the sequences shown in SEQ ID NOS:88-91.

18. An isolated DNA molecule comprising the sequences shown in SEQ ID NOS:92-98.

19. The DNA molecule according to claims 16, 17 or 18, wherein the molecule contains binding site for the CTCF protein.

20. The DNA molecule according to claims 16, 17 or 18, wherein the enhancer-blocking activity of the molecule is dependent upon CTCF binding to the molecule.

21. The DNA molecule according to claims 16, 17 or 18, wherein methylation of the cytosines (C) of the CpG residues in the molecule prevents CTCF binding to the molecule and inhibits the enhancer-blocking function of the molecule.

22. A method of activating the expression of an introduced gene from cis-acting DNA regulatory sequences in the chromatin into which the gene has integrated, comprising:

a) introducing into a cell a first DNA construct comprising a transcription unit comprising an expressible gene, a promoter to drive the expression of the gene, an enhancer element and an insulator element;

b) introducing into a cell a second DNA construct encoding a fusion protein, said fusion protein comprising the enzymatic domain of a methylase and the DNA binding domain of a DNA binding protein.

23. The method according to claim 22, wherein the first DNA construct further comprises DNA binding sequences for the DNA binding protein encoded by the second construct.

24. The method according to claim 22, wherein the DNA binding protein is Gal4 or LexA.

25. The method according to claim 22, wherein the methylase methylates the cytosines of the CpG residues in the insulator element.

26. The method according to claim 22, wherein the methylase is DNA methyltransferase 3.

27. The method according to claim 22, wherein the insulator element comprises sequences selected from the group consisted of SEQ ID NOS:84-100.

PCT

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Published
With international search report.

(54) Title: CHIMERIC DNA-BINDING/DNA METHYLTRANSFERASE NUCLEIC ACID AND POLYPEPTIDE AND USES THEREOF

(57) Abstract

The present invention provides a chimeric protein which comprises a mutated DNA methyltransferase portion and a DNA binding protein portion that binds sufficiently close to a promoter sequence of a target gene which promoter sequence contains a methylation site, to specifically methylate the site and inhibit activity of the promoter and thus inhibit expression of the target gene. This invention also provides for a method for inhibiting the expression of a target gene which includes contacting a promoter of the target gene with the chimeric protein, so as to specifically methylate the promoter sequence of the target gene thus inhibiting expression of the target gene.

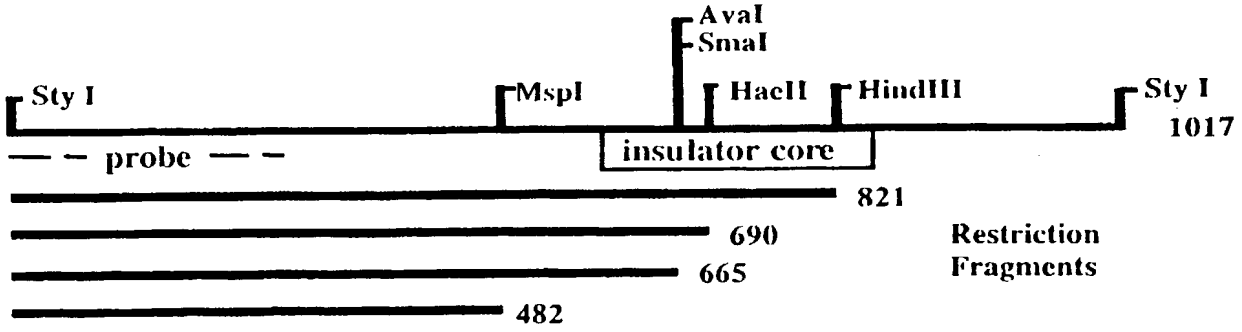


FIG. 1A

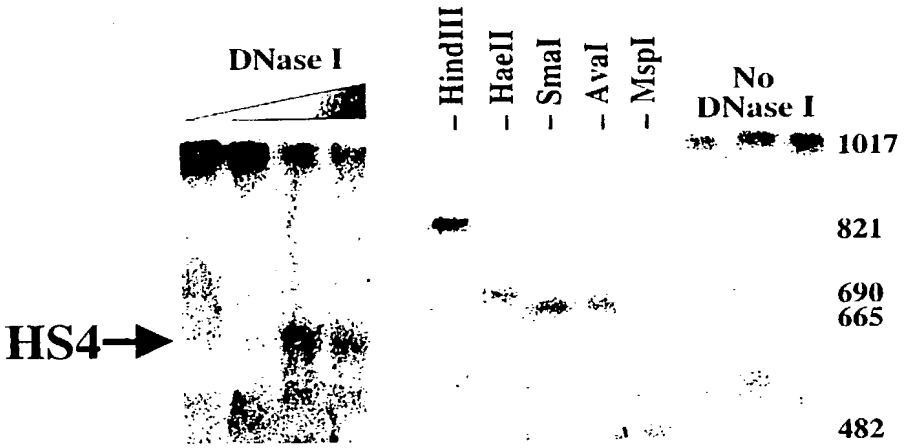


FIG. 1B

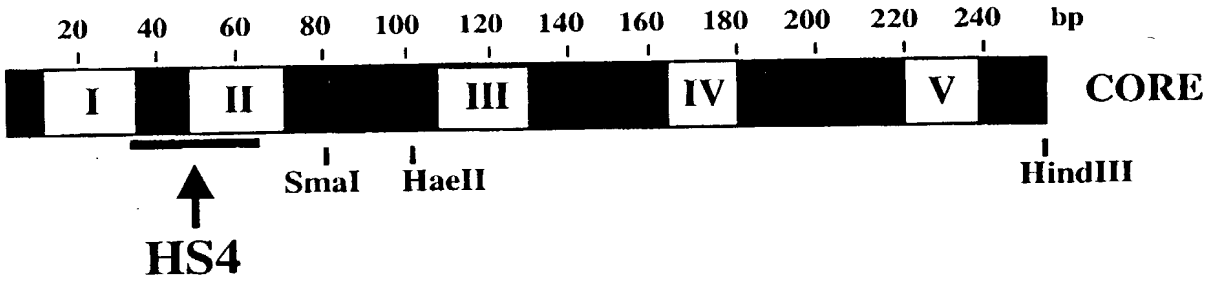
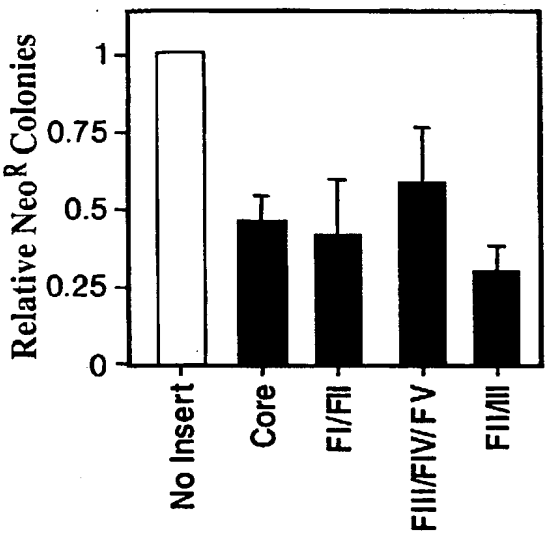


FIG. 1C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0.5
0.25
0
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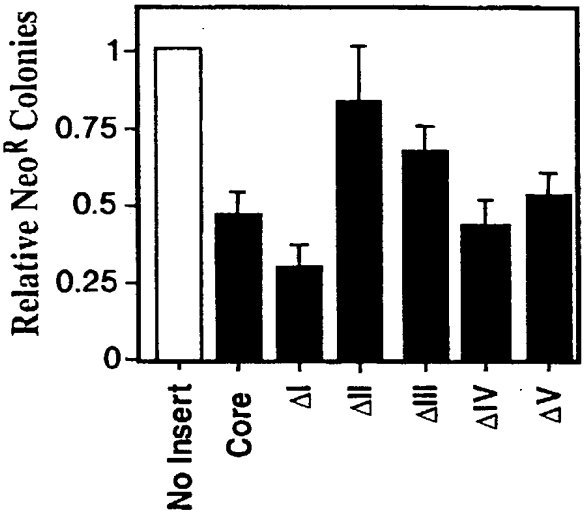
Core
FI/FII
FIII/FIV/FV
FII/III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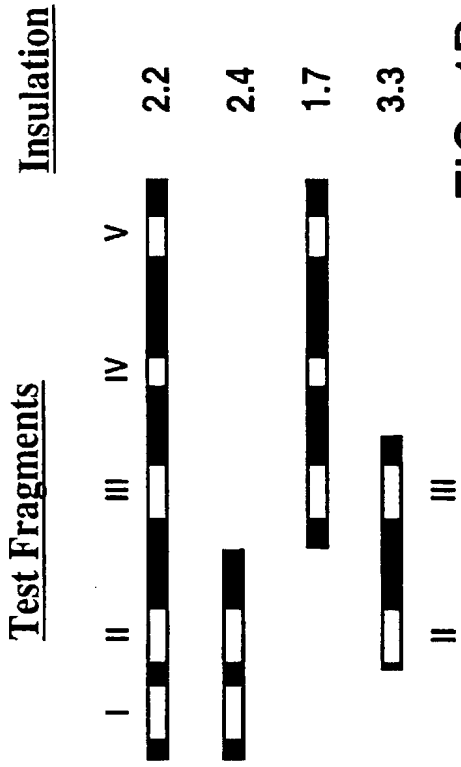
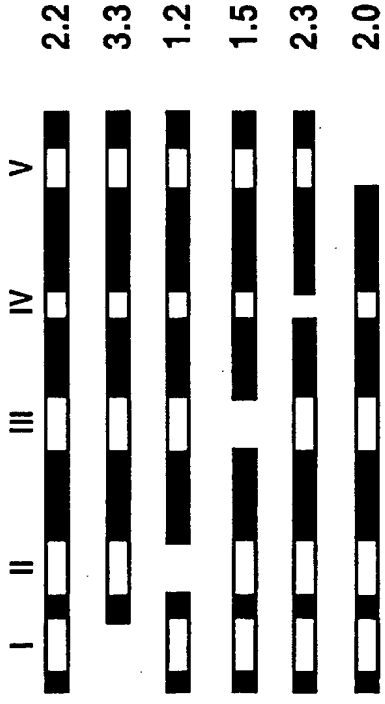
0.5
0.25
0
No Insert
Core
ΔI
ΔII
ΔIII
ΔIV
ΔV

FIG. 1D


Fold Insulation
Test Fragments
2.2
2.4
1.7
3.3
I
II
III
IV
V


2.2
3.3
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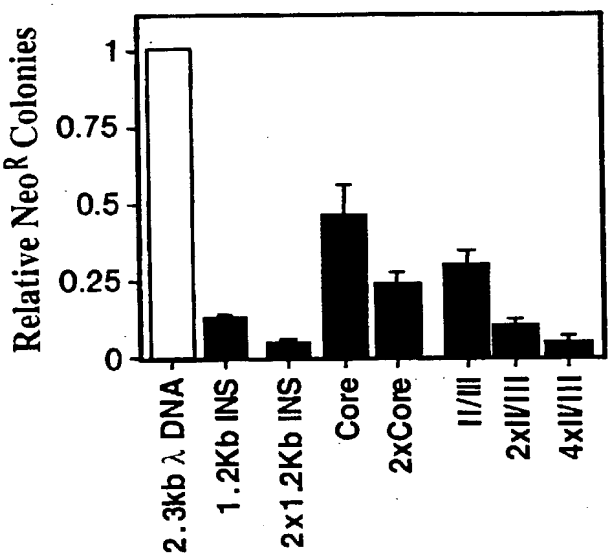
2.3
2.0
I
II
III
IV
V

FIG. 1E


Relative Neo^R Colonies
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0.75
0.5
0.25
0
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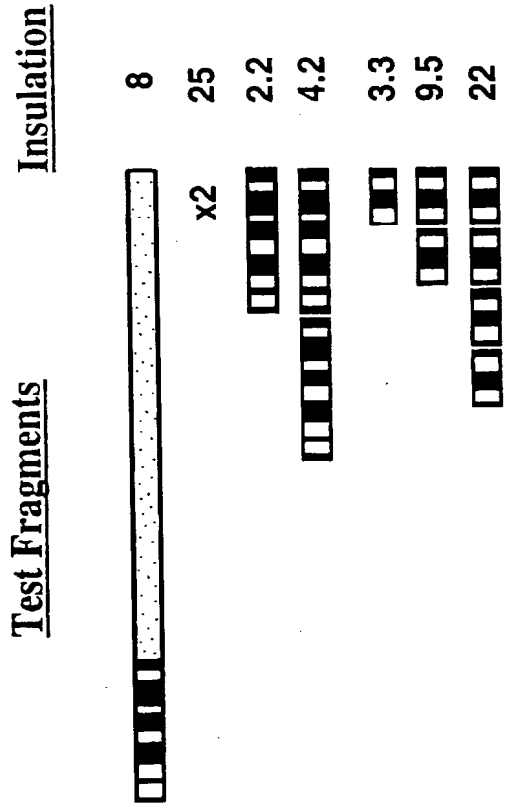
1.2Kb INS
2x1.2Kb INS
Core
2xCore
II/III
2xII/III
4xII/III

FIG. 1F


Test Fragments
Fold Insulation
x2
8
25
2.2
4.2
3.3
9.5
22

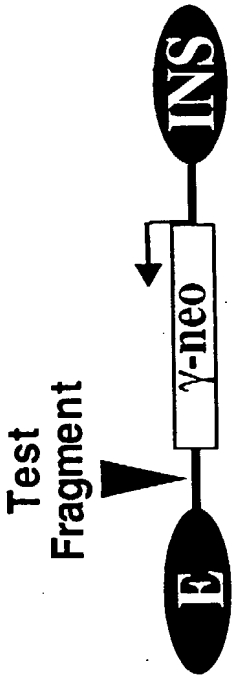

Test Fragment
E
γ-neo
INS

FIG. 1G

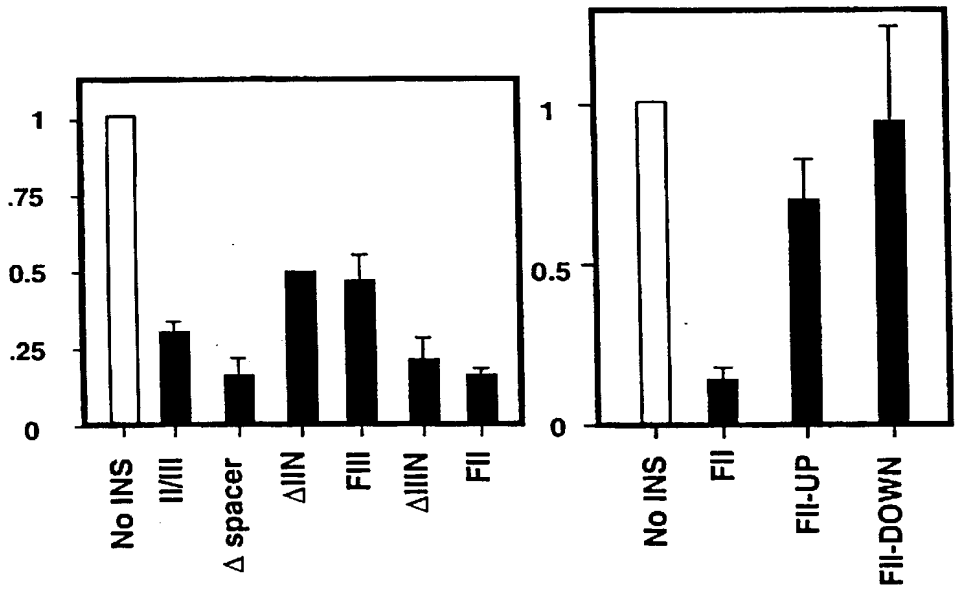


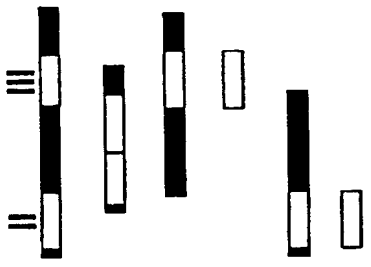
FIG. 2A

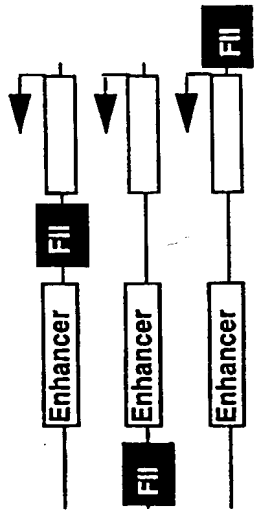
FIG. 2B

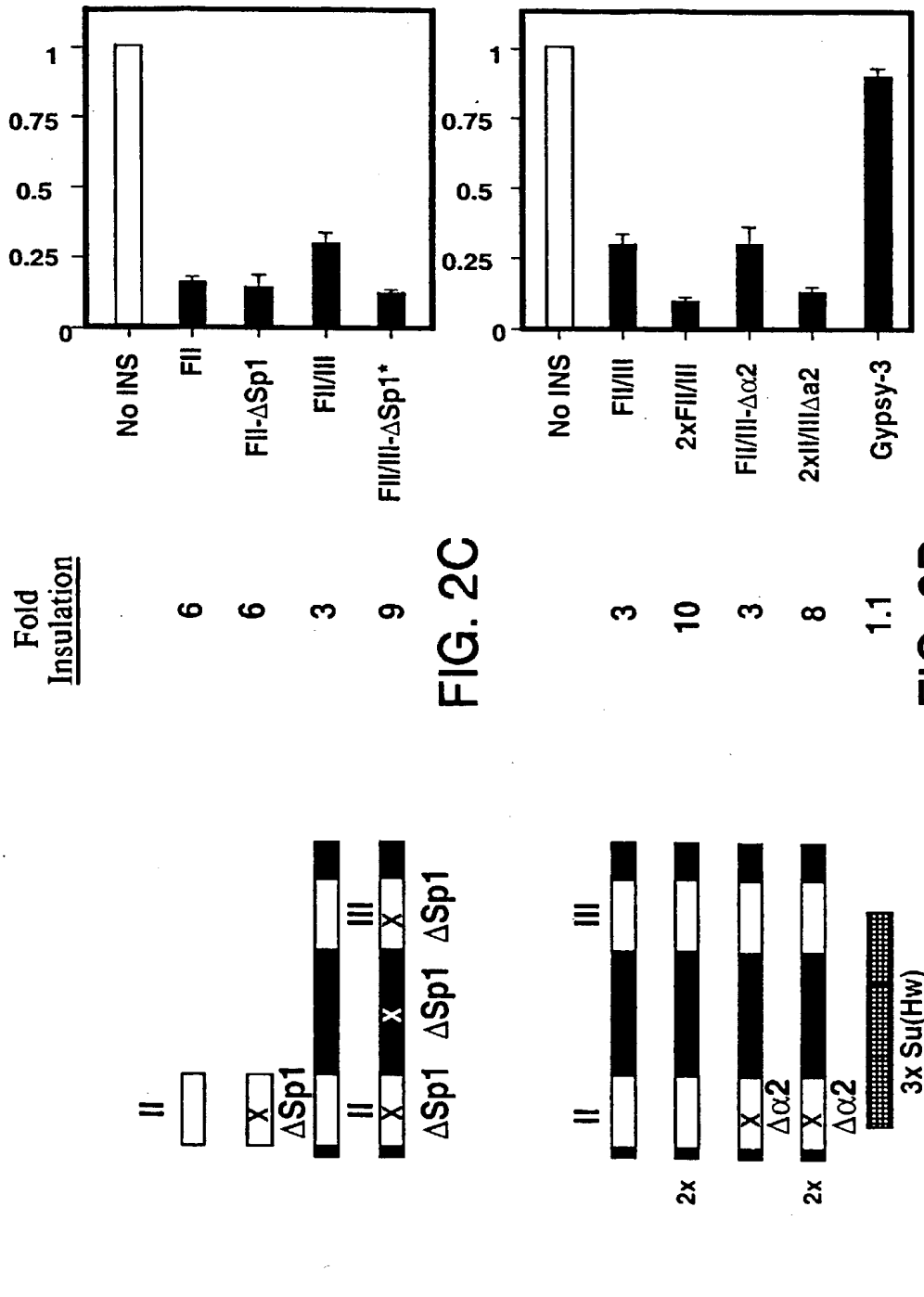
Fold Insulation

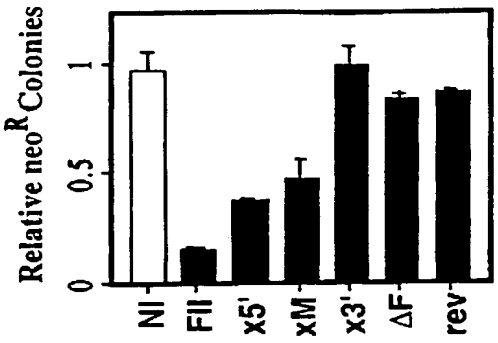
3.3
6.4
2.0
2.2
5.0
6.0

9
1.4
1.1



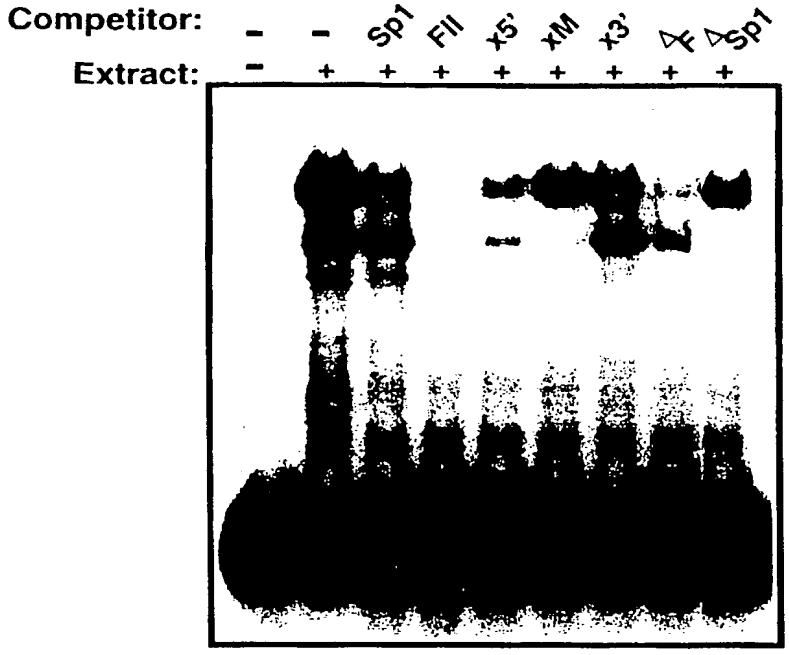



Fold Insulation
6
6
3
9
No INS
FII
FII-ΔSp1
FII/III
FII/III-ΔSp1*
1
0.75
0.5
0.25
0
II
X
ΔSp1
III
X
ΔSp1 ΔSp1 ΔSp1
FIG. 2C
3
10
3
8
1.1
No INS
FII/III
2xFII/III
FII/III-Δα2
2xII/IIIΔa2
Gypsy-3
2x
Δα2
3x Su(Hw)
FIG. 2D
SEQIDNO.2 5′-GGGATGTAATTACGTCCCTCCCCCGCTAGGGGGC-3′
3′-CCCTACATTAATGCAGGGAGGGGGCGATCCCCCG-5′
FII
SEQIDNO.3 Su(Hw) CCATACGTTRY GGGCGGGG Sp1 SEQIDNO.4
α2 TACATTAATGCA SEQIDNO.5
FIG. 2E



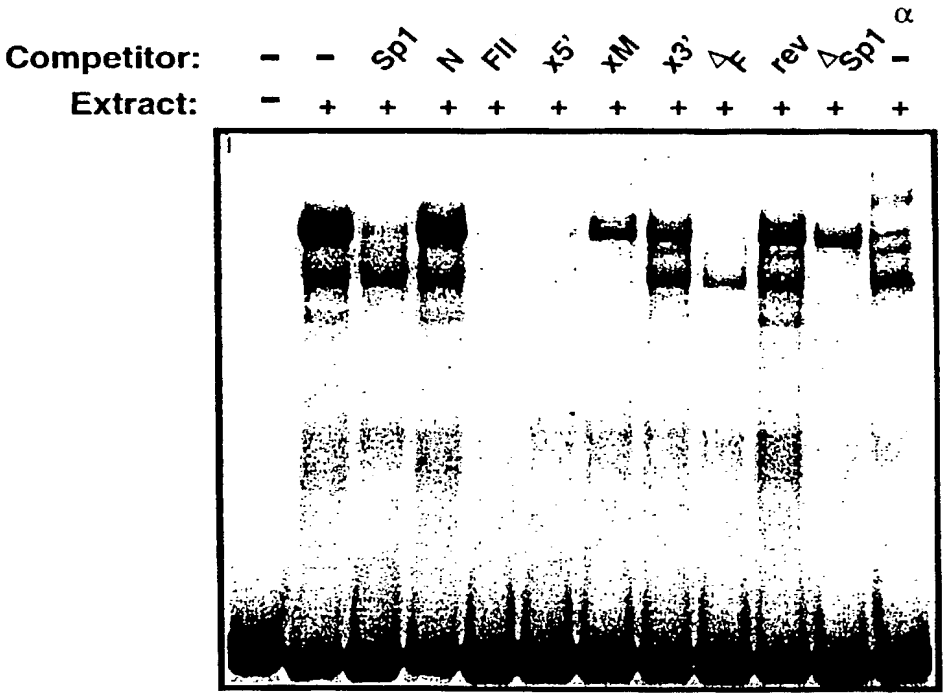
		Fold Insulation
SEQIDNO.1	CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA	-
SEQIDNO.6	CCCAtttcgtgccggcCGTCCCTCCCCCGCTAGGGGGCAGCA	6
SEQIDNO.7	CCCAGGGATGTAATTAatgaaagaaCCCGCTAGGGGGCAGCA	3
SEQIDNO.8	CCCAGGGATGTAATTACGTCCCTCCaaatagctttttCAGCA	2
SEQIDNO.9	GTAATTACGTCCCTCCCCCGCTA	-
SEQIDNO.10	ACGACGGGGGATCGCCCCCCTCCCTGCATTAATGTAGGGACCC	1.2
		1.1

FIG. 3A



K562

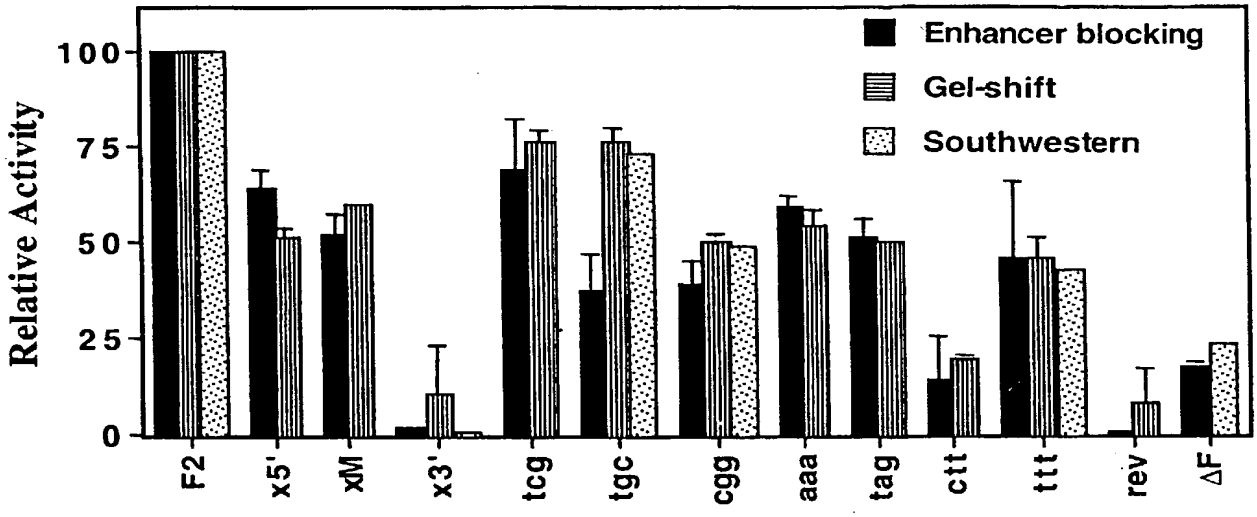
FIG. 3B



Chicken RBC

FIG. 3C

FIG. 3D



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SEQIDNO.6 CCCAtttcgtgccggcCGTCCCTCCCCCGCTAGGGGGCAGCA

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SEQIDNO.8 CCCAGGGATGTAATTACGTCCCTCCaaatagcttttttCAGCA

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SEQIDNO.12 CCCAGGGATtgcATTACGTCCCTCCCCCGCTAGGGGGCAGCA

SEQIDNO.13 CCCAGGGATGTAcggACGTCCCTCCCCCGCTAGGGGGCAGCA

SEQIDNO.14 CCCAGGGATGTAATTACGTCCCTCCaaaGCTAGGGGGCAGCA

SEQIDNO.15 CCCAGGGATGTAATTACGTCCCTCCCCCCtagAGGGGGCAGCA

SEQIDNO.16 CCCAGGGATGTAATTACGTCCCTCCCCCGCTcttGGGCAGCA

SEQIDNO.17 CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGttcCAGCA

SEQIDNO.10 ACGACGGGGGATCGCCCCCTCCCTGCATTAATGTAGGGACCC

SEQIDNO.9 GTAATTACGTCCCTCCCCCGCTA

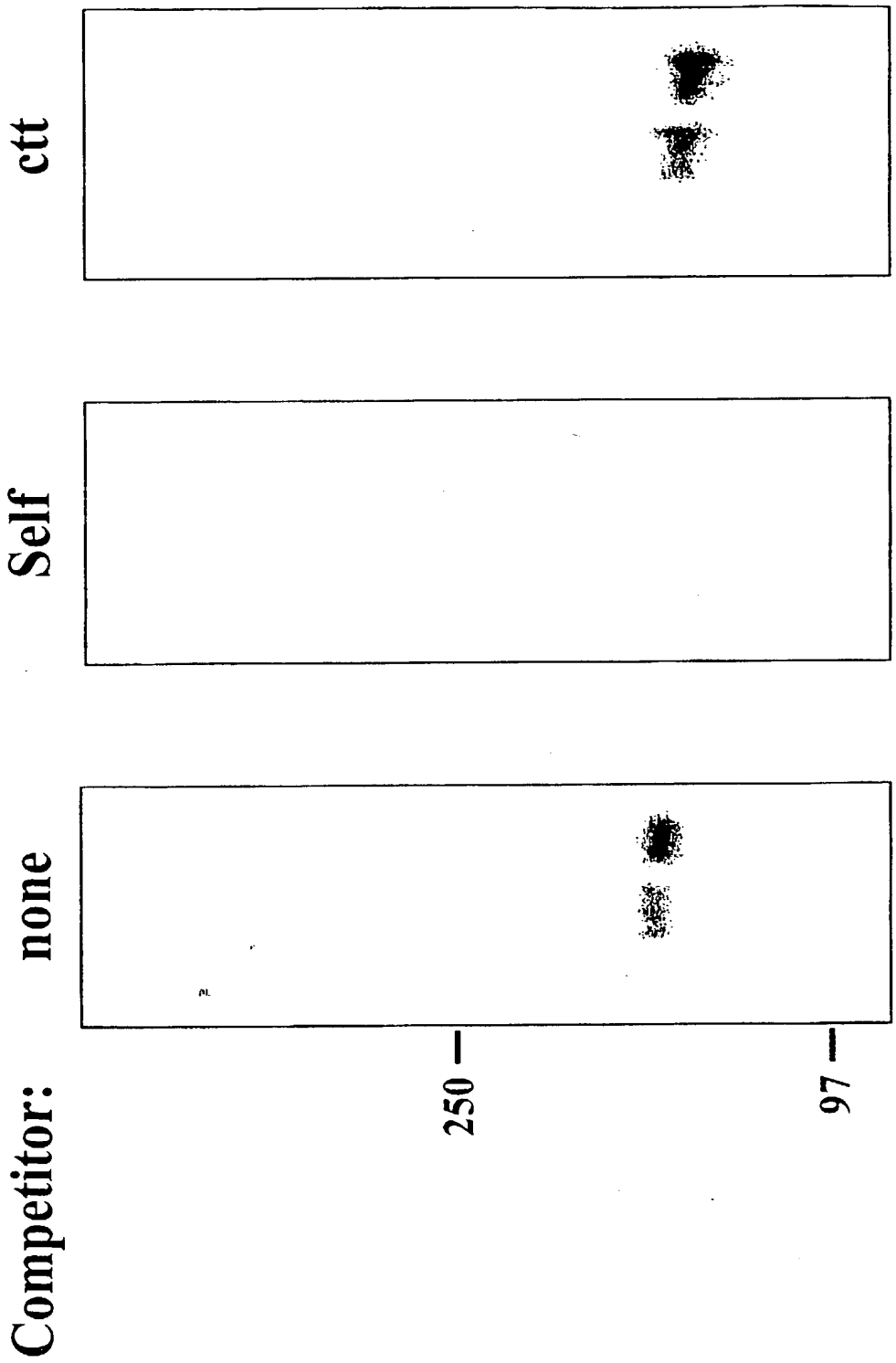


FIG. 4A

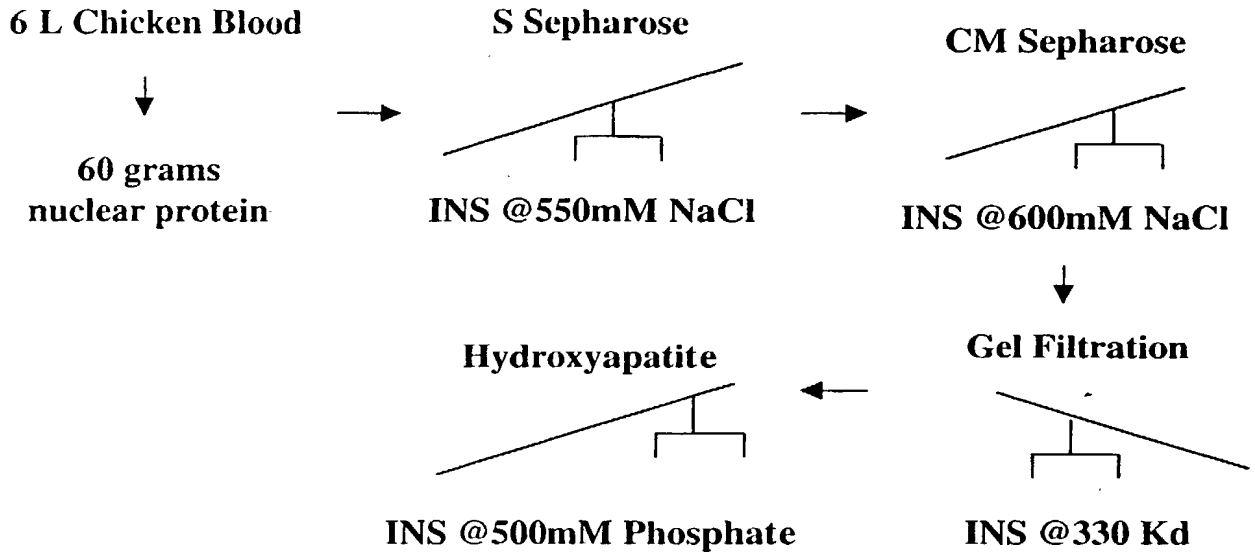
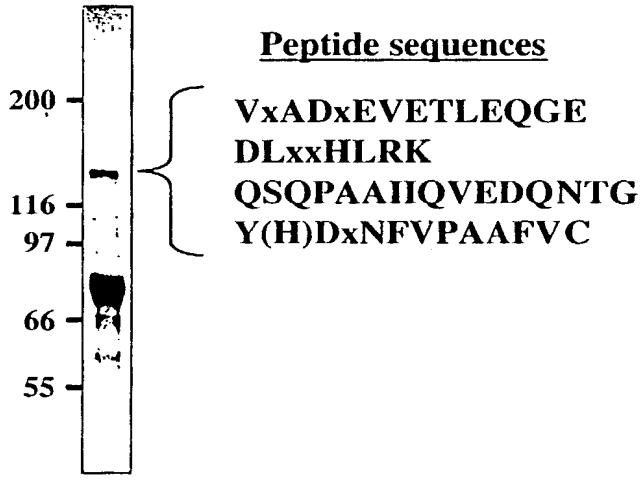
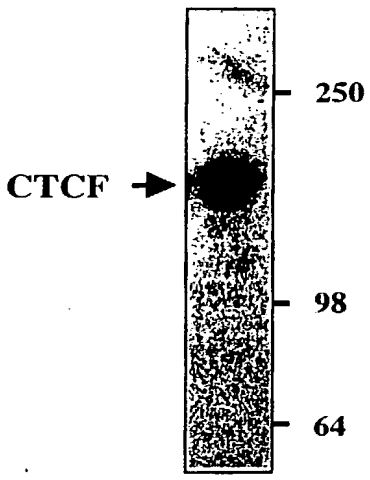


FIG. 4B



Coomassie

FIG. 4C



Southwestern

FIG. 4D

CTCF site	Fold insulation
FII	6±2
myc	4±2
lys	4±2
Apβ	7±1

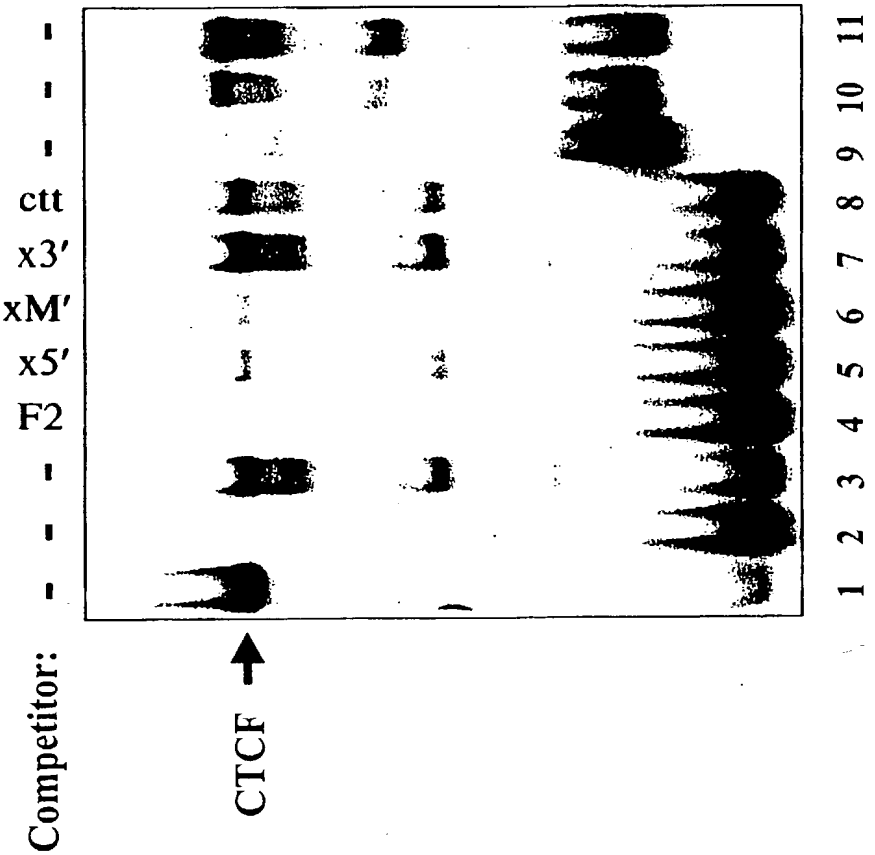


FIG. 5

```
FII      CCCAGGG--ATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCA   SEQIDNO.1
mycFV    CGCGGGCTCCGTGAGCGGGGAGGGCGCGCCGCGAGGGGGCGGCC   SEQIDNO.18
lys      AGCGGGCGCAGTTCCCCGGCGG----CGCCGCTAGGGTCTCTC   SEQIDNO.19
Apβ      CAAAAAGACATGTAAATACCATAGCTATCCAGTAGAGGTCTCAA   SEQIDNO.20

FIIx3'   CCCAGGG--ATGTAATTACGTCCCTCCaaatagctttttcAGCA   SEQIDNO.8
```

FIG. 6A

```
FII      CCCAGGGATGTAATTACGTCCCT-----CCCCCGCTAGGGGGCAGCA   SEQIDNO.1
RO 1...ACCCGATTCGGGGTCGGGGCCCCGGGGGGTGCCCGCTAAGGGGCCCCG...SEQIDNO.21
RO 2...CCCGATTCGGGGTCGGGGCCCCGGGGGGTGCCCGC--GGGGGCCCCGG...SEQIDNO.22
RO 3...ACCCGATTCGGGGTCGGGGCCCCGGGGGGTGCCCGC--GGGGGCCCCG...SEQIDNO.23
RO 4...ACCCGATTCGGGGTCGGGGCCCCGGGGGGTGCCCGC--GGGGGCCCCG...SEQIDNO.24
RO 5...ACCCGATTCGGGGTCGGGGCCCCGGG---CCCCGC--GGGGGCCCCG...SEQIDNO.25
RO 6...ACCCGATTCGGGGTCGGGGCCCCGGGGGGTGCCCGC--GGGGGCCCCG...SEQIDNO.26
RO 7...ACCCGATTCGGGGTCGGGGCCCCGGGGGGTGCCCGCTAAGGGGCCCCG...SEQIDNO.27
```

FIG. 6B

```
FII      CCCAGGGATGTAATTACGTCCTCCCCCGCTAGGGGGCAGCA SEQIDNO.1
BEAD A   CCCAGGCCTGCACTGCCG--CCTGCCG-GCAGGGGTCCAGTC SEQIDNO.28
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FIG. 6C

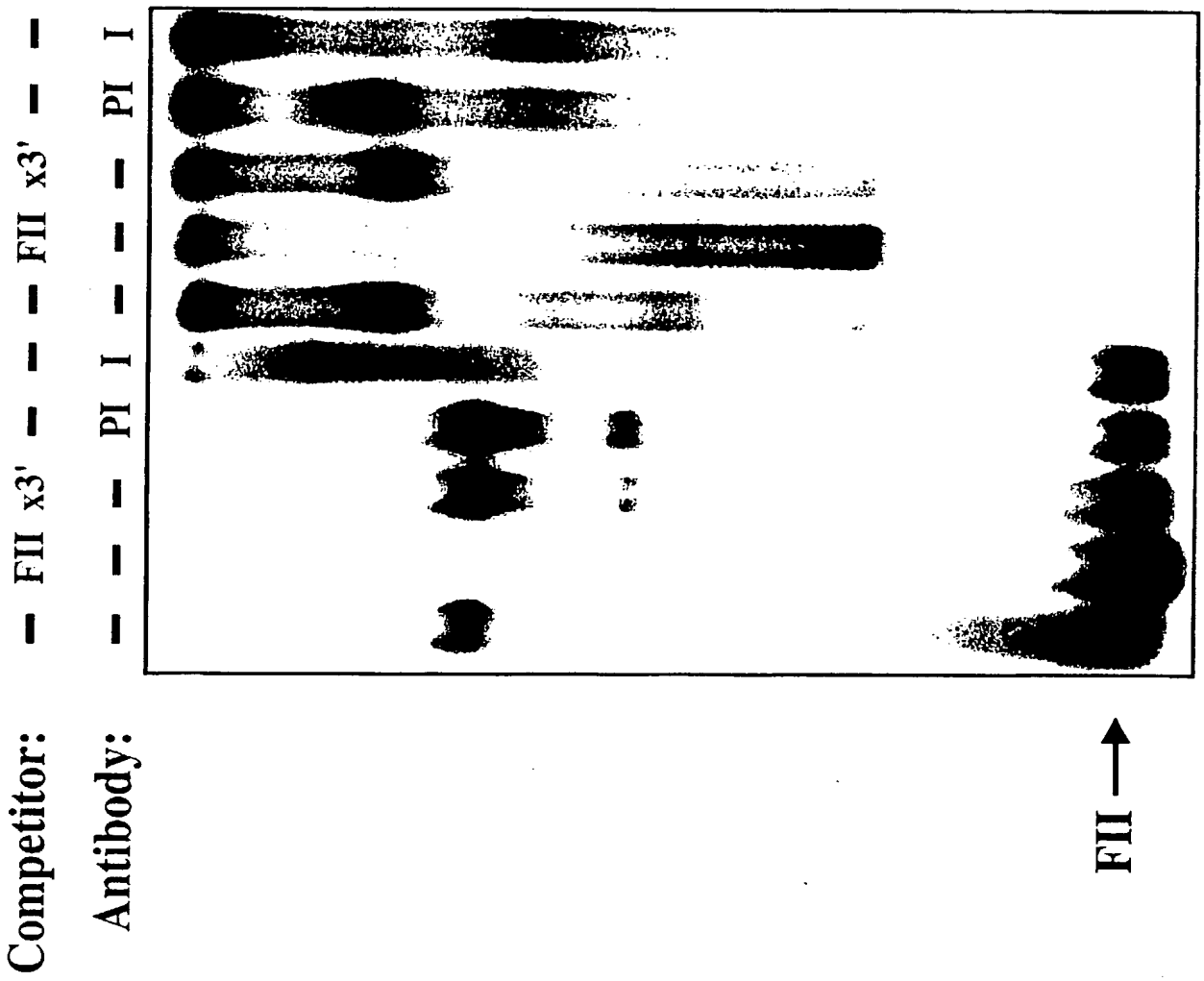
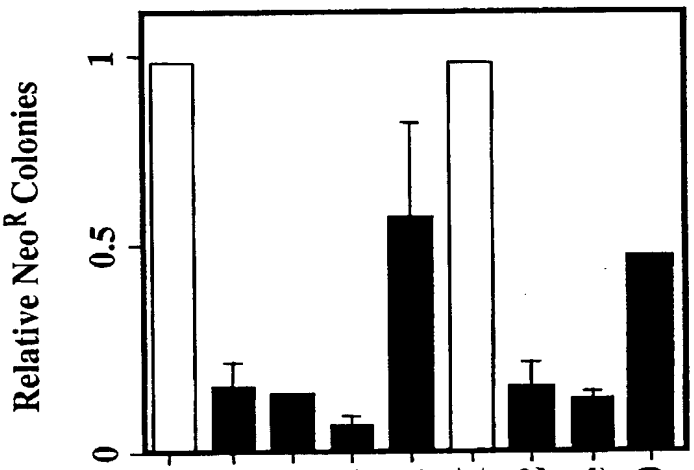
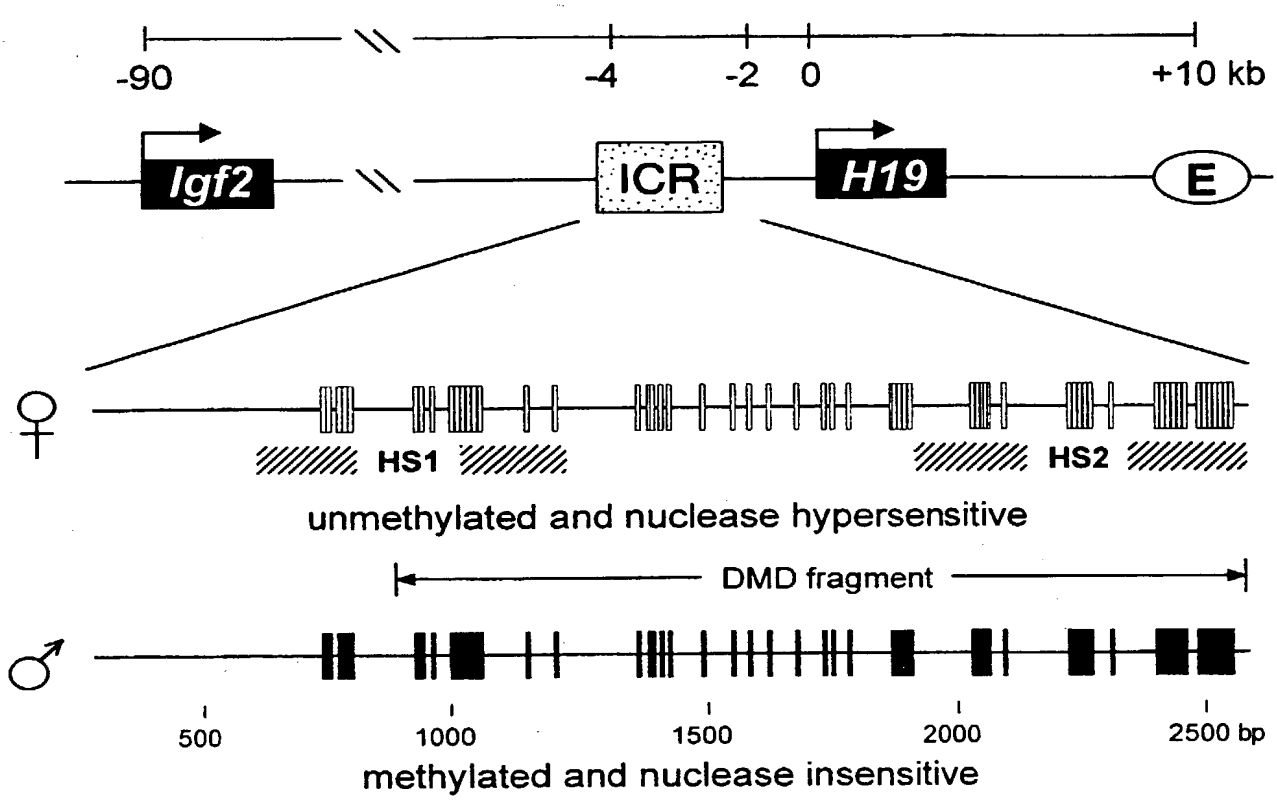


FIG. 7A



Fold Insulation
-
6
7
17
2
-
6
8
2

FIG. 7B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-2
0
+10 kb
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ICR
H19
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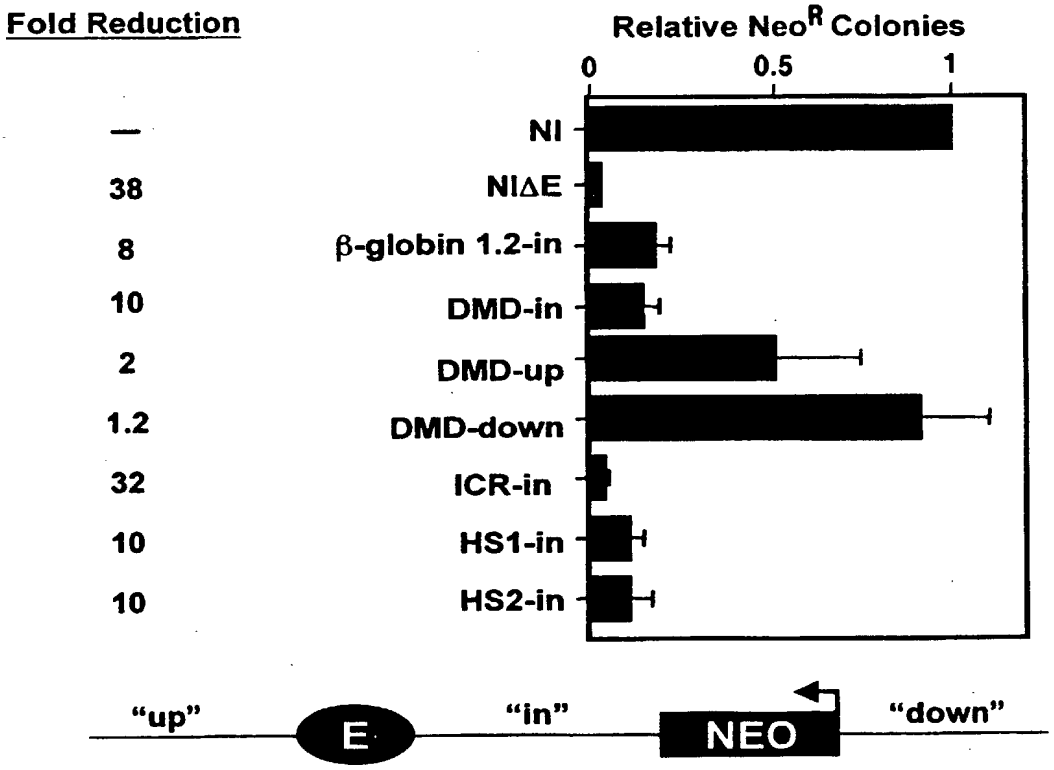
HS1
HS2
unmethylated and nuclease hypersensitive
DMD fragment
500
1000
1500
2000
2500 bp
methylated and nuclease insensitive

FIG. 8A


Fold Reduction
Relative Neo^R Colonies
0
0.5
1
—
NI
38
NIΔE
8
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10
DMD-in
2
DMD-up
1.2
DMD-down
32
ICR-in
10
HS1-in
10
HS2-in
"up"
E
"in"
NEO
"down"

FIG. 8B

```
                m1 GTTGTGGGGTTTATACGCGGGAGTTGCCGCGTGGTGGCAGCAAAATCG SEQIDNO.84
      Mouse     m2 TTTGTGTG---TAAAGACCAGGGTTGCCGCACGGCGGCAGTGAAGTCT SEQIDNO.85
Igf2-H19        m3 TATGTGCAACAAGGGAACGGATGCTACCGCGCGGTGGCAGCATACTCC SEQIDNO.86
                m4 GCTATA-GCTAATGGACAGACGATGCCGCGTGGTGGCAGTACAATAC SEQIDNO.87

                r1 TTGTGTGGTTTAAAACGCGGAAGTTGCCGCGTGGTGGCAGCAAAAATC SEQIDNO.88
       Rat      r2 TCCTTTGCGCGTAAAAACCAGGCCTGCCGCGTGGCGGCAGTGAAGTCG SEQIDNO.89
Igf2-H19        r3 TTGTGTGCACGGGGAAATGGATGTTACCGCGCGGTGGCAGCATACTCC SEQIDNO.90
                r4 TGACTATAGCTAGGATGGACAAATATGCCGCGTGGTGGCAGTACAACCC SEQIDNO.91

                h1 GGCTGTACGTGTGGAATCAGAAGTGGCCGCGCGGCGGCAGTGCAGGCT SEQIDNO.92
                h2 GGTTGTAGTTGTGGAATCGGAAGTGGCCGCGCGGCGGCAGTGCAGGCT SEQIDNO.93
      Human     h3 GGTTGTAGCTGTGGAATCGGAAGTGGCCGCGTGGCGGCAGTGCAGGCT SEQIDNO.94
Igf2-H19        h4 GGTTGTAAGTGTGGACTCAAAAGTGGCCGCGCGGCGGCAGTGCAGGCT SEQIDNO.95
                h5 GGTTGTAGTTGTGGAATCGGAGGTGGCTGCGCGGCGGCAGTGCAGGCT SEQIDNO.96
                h6 GGTTGTAGTTGTGGAATCGGAAGTGGCCGCGCGGCGGCAGTGCAGGCT SEQIDNO.97
                h7 GGTTGTGGCCTGTGGAGACGGAAATGGCCGAGAGGCGGCAGTGGTGACT SEQIDNO.98

                                CCGCGNGGNGGCAG SEQIDNO.99

   Igf2-H19
   consensus

β-globin  F11 CCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGGGCAGCAGCGAGC SEQIDNO.100
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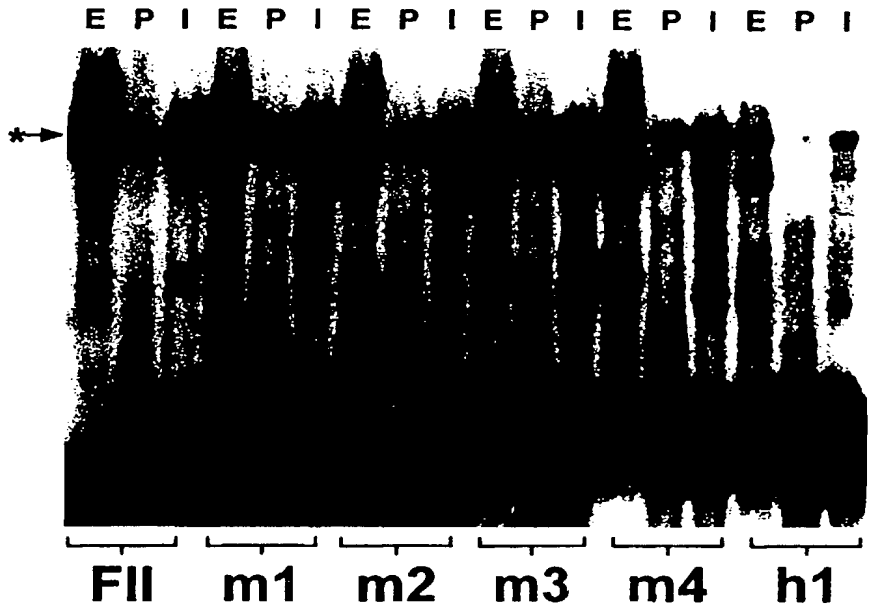
FIG. 9A

FIG. 9B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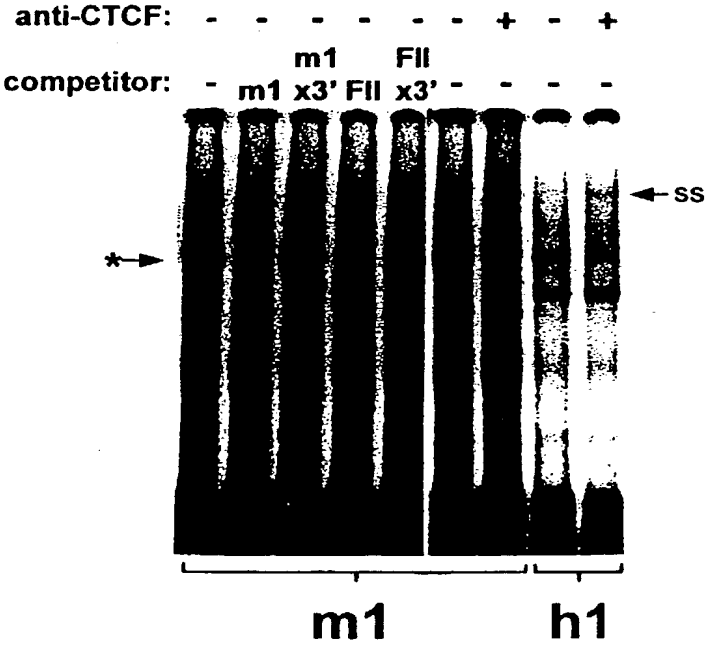
FII m1 m2 m3 m4 h1

FIG. 9C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competitor: - m1 m1 x3' FII FII x3' - - - -
ss
m1 h1

FIG. 9D

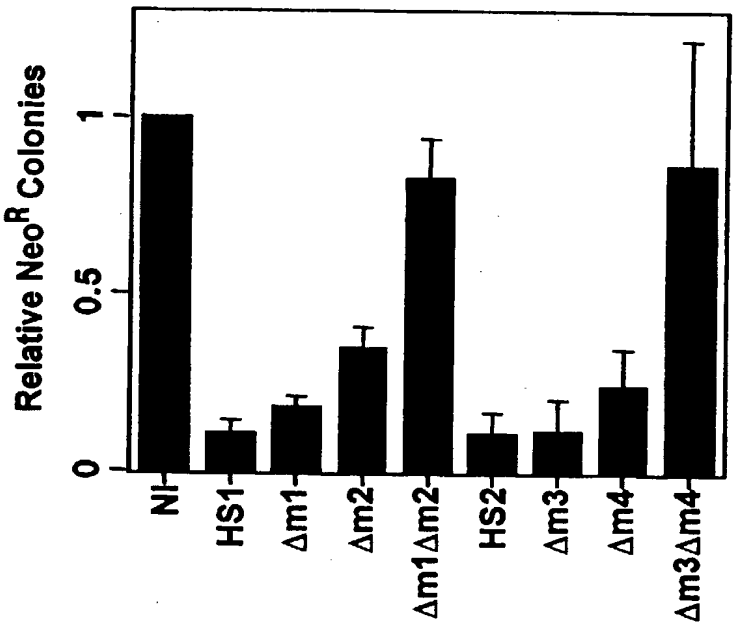


FIG. 10A

Fold Reduction	-	10	6	3	1.2	10	9	4	1.2

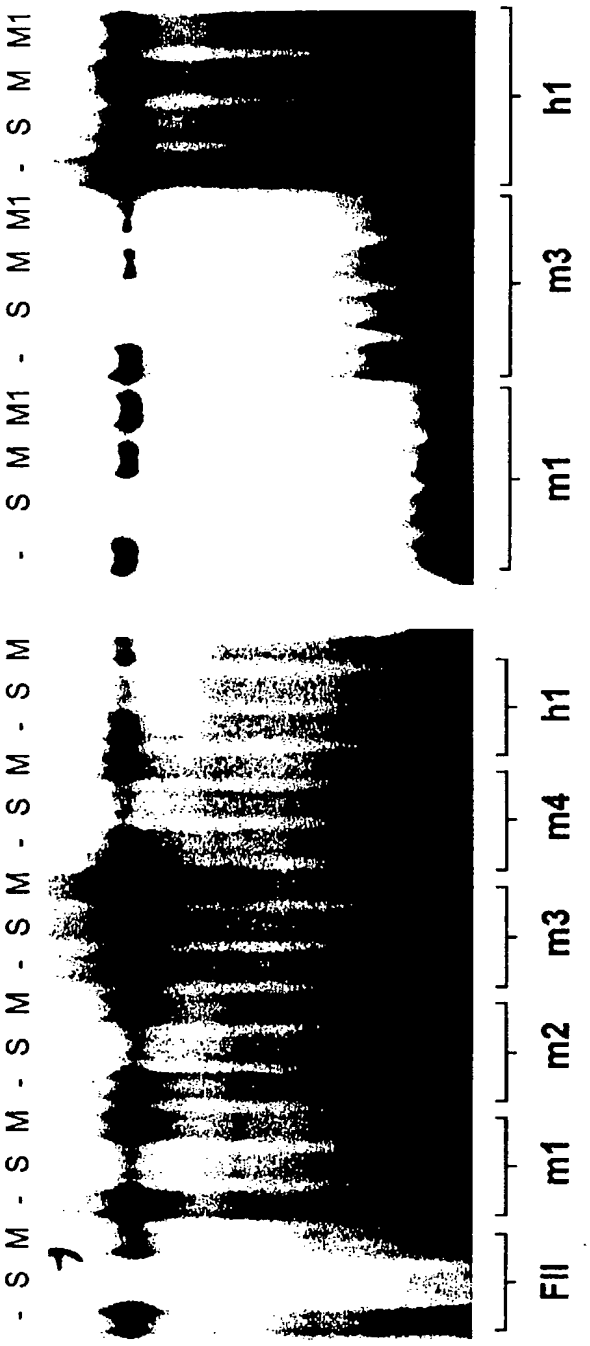
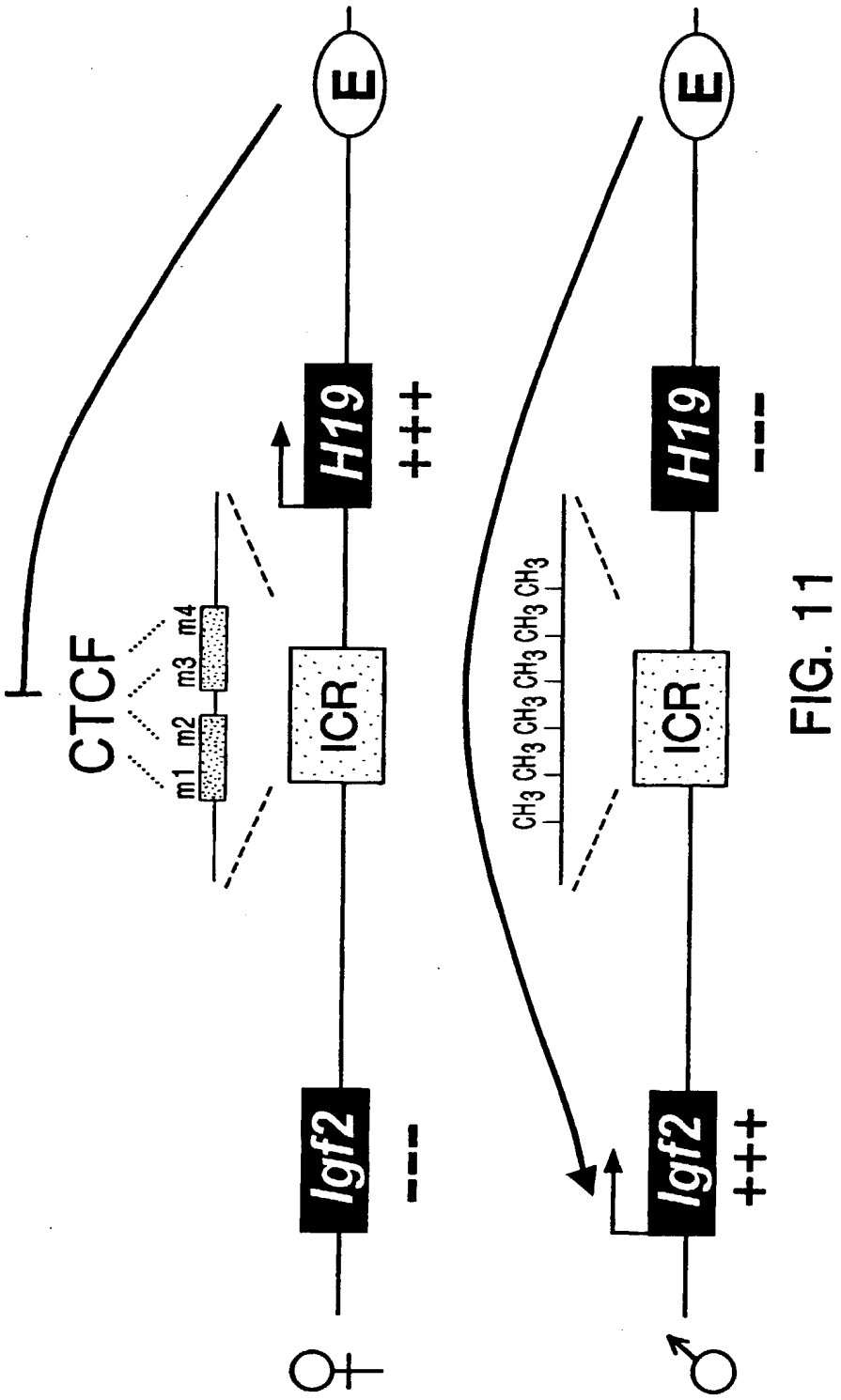


FIG. 10B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H19
+++
CTCF
m4
m3
m2
m1
ICR
Igf2

E
H19

CH3 CH3 CH3 CH3 CH3 CH3
ICR
Igf2
+++

FIG. 11

Docket No. 2026-4297US1

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, the information given herein is true, that I believe I am the original, first and sole (if only one name is listed below) or an or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

DNA BINDING PROTEIN AND SEQUENCE AS INSULATORS HAVING SPECIFIC ENHANCER BLOCKING ACTIVITY FOR REGULATION OF GENE EXPRESSION

which is described in:

[x] the specification in Application Serial No 10/019,386 filed December 28, 2001
(if applicable) and amended on _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to me which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56 (a).

I hereby claim foreign priority benefits under Title 35 United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign applications(s) for patent or inventor's certificate or any PCT international applications(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

COUNTRY	APPLICATION	DATE OF FILING (DAY, MONTH, YEAR)	PRIORITY CLAIMED UNDER 35 USC § 119
			[] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional patent application(s).

Docket No. 2026-4297US1

Provisional Application Serial No.	Filing Date	Status: patented, pending, abandoned
60/141,728	30 June 1999	Pending

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) or PCT International application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

US/PCT Application Serial No.	Filing Date	Status: patented, pending, abandoned
PCT/US00/10509	19 April 2000	Pending

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

(11)

James C. Haight (Reg. No. 25,588); David R. Sadowski (Reg. No. 32,808); Robert Benson (Reg. No. 33,612); Jack Spiegel (Reg. No. 34,477); Susan S. Rucker (Reg. No. 35,762); Stephen Finley (Reg. No. 36,357); Steven Ferguson (Reg. No. 38,448); John Peter Kim (Reg. No. 38,514); Norbert Pontzer (Reg. No. 40,777); Richard U. Rodriguez (Reg. No. 45,980) and Marlene Shinn (P-46,005) all of the Office of Technology Transfer, National Institutes of Health, 6011 Executive Boulevard, Suite 325, Rockville, Maryland 20852.

I further direct that all correspondence concerning this application be directed to:

Patent Branch
Office of Technology Transfer
National Institutes of Health
6011 Executive Boulevard, Suite 325
Rockville, MD 20852
Telephone: (301) 496-7056
Fax: (301) 402-0220

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Docket No. 2026-4297US1

Full Name of first joint inventor: Adam C. BELL

Inventor's signature: _____ Date: 10/02/02

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Post Office Address: 13312 Burnt Woods Place, Germantown, Maryland 20874

Full Name of second joint inventor: Adam G. WEST

Inventor's signature: _____ Date: _____

Country of Citizenship: United States

Residence: 4511 Everett Street, Kensington, Maryland 20985

Post Office Address: 4511 Everett Street, Kensington, Maryland 20985

Full Name of third joint inventor: Gary FELSENFELD

Inventor's signature: _____ Date: _____

Country of Citizenship: United States

Residence: 3109 Leland Street, Chevy Chase, Maryland 20815

Post Office Address: 3109 Leland Street, Chevy Chase, Maryland 20815

Docket No. 2026-4297US1

Full Name of first joint inventor: Adam C. BELL

Inventor's signature: _____ Date: _____

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Post Office Address: 18670 Winding Creek Place, Germantown, Maryland 20874

Full Name of second joint inventor: 2-DD Adam G. WEST

Inventor's signature: Adam West Date: 10/2/02

Country of Citizenship: ~~United States~~ KINGDOM GBX

Residence: 4511 Everett Street, Kensington, Maryland 20985 USX

Post Office Address: 4511 Everett Street, Kensington, Maryland 20985

Full Name of third joint inventor: 3-DD Gary FELSENFELD

Inventor's signature: Gary Felsenfeld Date: 10-2-02

Country of Citizenship: United States USX

Residence: 3109 Leland Street, Chevy Chase, Maryland 20815

Post Office Address: 3109 Leland Street, Chevy Chase, Maryland 20815

SEQUENCE LISTING

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Felsenfeld, Gary

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<210> 63
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<110> Bell, Adam
West, Adam
Felsenfeld, Gary

<120> DNA BINDING PROTEIN AND SEQUENCE AS INSULATORS HAVING SPECIFIC ENHANCER BLOCKING ACTIVITY FOR REGULATION OF GENE EXPRESSION

<130> 20264297PC

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<150> 60/141,728
<151> 1999-06-30

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cgagccgccc ggggctccgc ggcgcgcct 89

<210> 51
<211> 60
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:primer

<400> 51
aggcgcgccc ccagggatgt aattacgtcc ctccccccgct agggggcagc aggcgcgcct 60

<210> 52
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 52
aggcgcgccc cggtccggcg ctcccccccgc atccccgagc cggggcgcgc ct 52

<210> 53
<211> 98
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 53
aggcgcgcca aaatacattg cataccctct tttaataaaa aatattgcat acgttgacga 60
aacaaatttt cgttgcatac ccaataaaag gcgcgcct 98

<210> 54
<211> 98
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 54
aggcgcgccg gggggggggca cggagcccct cggccgcccc ctcgcggcgc gccctccccg 60
ctcacggagc ccgcgcggag ccgggggcga ggcgcgcc 98

<210> 55
<211> 97
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 55
aggcgcgcct ttagctgcat ttgacatgaa gaaattgaga cctctactgg atagctatgg 60
tatttacatg tctttttgct tagttactag gcgcgcc 97

<210> 56
<211> 98
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 56
aggcgcgccc cctcccggcg cgagcgggcg cagttccccg gcggcgccgc taggggtctc 60
tctcgggtgc cgagcggggt gggccggata ggcgcgcc 98

<210> 57
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 57
aggcgcgccg gggacccgat tcggggtcgg ggccccgggg gtgcccgcta aggggccccg 60
gggggccctc ccggcgaaga ggggcccatt ggcgcgcct 99

<210> 58
<211> 137
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 58
ggcgcgccgt ggaagaggga tgttgagggc ccaggggctg ccttgccggt gcattggctg 60
cccaggcctg cactgccgcc tgccggcagg ggtccagtcc acgagaccca gctccctgct 120
ggcggaaggg cgcgcct 137

<210> 59
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 59
Ala Pro Asn Gly Asp Leu Thr Pro Glu Met Ile Leu Ser Met Met Asp
1 5 10 15

<210> 60
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 60
cccagggatg taattacgtc cctcccccgc taggggcag caggcgcgcc t 51

<210> 61
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 61
aggcgcgcct gctgc 15

<210> 62
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 62
aggcgcgcca agctttgtca cagcggaccc caacctatg 39

<210> 63
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 63
aggcgcgccc agagctcttt ctccaccact tgtctaagt 39

<210> 64
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 64
aggcgcgccg gtacctcgtg gactcggact cccaaatca 39

<210> 65
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 65
aggcgcgcca tagtagctat acttcaattt tca 33

<210> 66
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 66
aggcgcgcct ttataagagg ttggaacact tgt 33

<210> 67
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 67
ccctattctt ggacgtctgc tgaatctatt ggaattcaca aatggcaatg c 51

<210> 68
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 68
gattcagcag acgtccaaga ataggg 26

<210> 69
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 69
gactcggact cccaaatcaa caaggacgga ttgcaactga ttgagttttc 50

<210> 70
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 70
ccttgttgat ttgggagtcc gagtc 25

<210> 71
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 71
aggcgcgcca agactgaagg agctacccaa gaa 33

<210> 72
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 72
aggcgcgcct ttataagagg ttggaacact tgt 33

<210> 73
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 73
aggcgcgcca gagaacttga ctcattccct acac 34

<210> 74
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 74
agaagctgtt atgtgcaaca agggagcgat tcattcccag caatatcc 48

<210> 75
<211> 24
<212> DNA
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<220>
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<400> 75
cccttgttgc acataacagc ttct 24

<210> 76
<211> 88
<212> DNA
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aggcgcgccg ttgtggggtt tatacgcggg agttgccgcg tggtggcagc aaaatcgatt 60
gcgccaaacc taaagagccg gcgcgcct 88

<210> 77
<211> 83
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aggcgcgcca atcctttgtg tgtaaagacc agggttgccg cacggcggca gtgaagtctc 60
gtacatcgca gtccggcgcg cct 83

<210> 78
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<400> 78
aggcgcgccc tgttatgtgc aacaagggaa cggatgctac cgcgcggtgg cagcatactc 60
ctatatatcg tggcccaaag gcgcgcct 88

<210> 79
<211> 88
<212> DNA
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<400> 79
aggcgcgcca cgctgtgcag atttggctat agctaaatgg acagacgatg ccgcgtggtg 60
gcagtacaat actacatatg gcgcgcct 88

<210> 80
<211> 91
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 80
gccctgatgg cgcagaatcg gctgtacgtg tggaatcaga agtggccgcg cggcggcagt 60
gcaggctcac acatcacagc ccgagcacgc c 91

<210> 81
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 81
aggcgcgccc ccagggatgt aattacgtcc ctccaaatag ctttttcagc aggcgcgcct 60

<210> 82
<211> 88
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 82
aggcgcgcct gctgaatcag ttgtggggtt tatacgcggg agttgaatat gttgttactc 60
aaaatcgatt gcgccaaacg gcgcgcct 88

<210> 83
<211> 102
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 83
gctgttatgt gcaacaaggg aacggatgct taccgcgcgg tggcagcata ctcctatata 60
tcgtggccca aatgctgcca acttgggggg agcgattcat tc 102

<210> 84
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
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<400> 84
gttgtggggt ttatacgcgg gagttgccgc gtggtggcag caaaatcg 48

<210> 85
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 85
tttgtgtgta aagaccaggg ttgccgcacg gcggcagtga agtct 45

<210> 86
<211> 48
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 86
tatgtgcaac aagggaacgg atgctaccgc gcggtggcag catactcc 48

<210> 87
<211> 47
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 87
gctatagcta aatggacaga cgatgccgcg tggtggcagt acaatac 47

<210> 88
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 88
ttgtgtggtt taaaacgcgg aagttgccgc gtggtggcag caaaaatc 48

<210> 89
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 89
tcctttgcgc gtaaaaacca ggcctgccgc gtggcggcag tgaagtcg 48

<210> 90
<211> 48
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 90
ttgtgtgcac ggggaaatgg atgttaccgc gcggtggcag catactcc 48

<210> 91
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 91
tgactatagc tagatggaca aatatgccgc gtggtggcag tacaaccc 48

<210> 92
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 92
ggctgtacgt gtggaatcag aagtggccgc gcggcggcag tgcaggct 48

<210> 93
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 93
ggttgtagtt gtggaatcgg aagtggccgc gcggcggcag tgcaggct 48

<210> 94
<211> 48
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 94
ggttgtagct gtggaatcgg aagtggccgc gtggcggcag tgcaggct 48

<210> 95
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 95
ggttgtaagt gtggactcaa aagtggccgc gcggcggcag tgcaggct 48

<210> 96
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 96
ggttgtagtt gtggaatcgg aggtggctgc gcggcggcag tgcaggct 48

<210> 97
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 97
ggttgtagtt gtggaatcgg aagtggccgc gcggcggcag tgcaggct 48

<210> 98
<211> 48
<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:primer

<400> 98
ggttgtggct gtggagacgg aaatggccga gaggcggcag tggtgact 48

<210> 99
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<220>
<223> The "n" at positions 6 and 9 can be either C or T.

<400> 99
ccgcgnggng gcag 14

<210> 100
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer

<400> 100
cccagggatg taattacgtc cctcccccgc taggggcag cagcgagc 48

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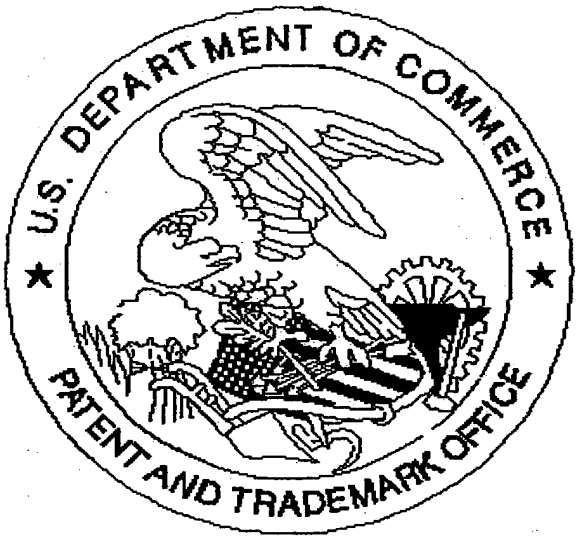

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